US005262035A

United States Patent [19]

Gregg et al.

[11] Patent Number: 5,262,035

[45] Date of Patent: Nov. 16, 1993

[54] ENZYME ELECTRODES

[75] Inventors: Brian A. Gregg; Adam Heller, both of Austin, Tex.

[73] Assignee: E. Heller and Company, Austin, Tex.

[21] Appl. No.: 389,226

[22] Filed: Aug. 2, 1989

[51] Int. Cl.[5] G01N 27/327

[52] U.S. Cl. 204/403; 204/153.12; 435/817; 436/518; 436/531; 436/806

[58] Field of Search 204/153.12, 403; 435/817; 436/806, 518, 531

[56] References Cited

U.S. PATENT DOCUMENTS

4,224,125	9/1980	Nakamura et al.	435/817
4,461,691	7/1984	Frank	204/290 R
4,476,003	10/1984	Frank et al.	204/290 R
4,524,114	6/1985	Samuels et al.	204/294
4,545,382	10/1985	Higgins et al.	204/403
4,619,754	10/1986	Niki et al.	204/290 R
4,655,885	4/1987	Hill et al.	204/290 R
4,711,245	12/1987	Higgins et al.	204/415
4,717,673	1/1988	Wrighton et al.	204/290 F
4,721,601	1/1988	Wrighton et al.	204/435
4,764,416	8/1988	Ueyama et al.	428/212
4,784,736	11/1988	Lonsdale et al.	204/157.15
4,894,339	1/1990	Hawazato et al.	435/817
4,917,800	4/1990	Lonsdale et al.	210/490
4,968,400	11/1990	Shimomura et al.	204/403
5,089,112	2/1992	Skotheim et al.	204/153.12

FOREIGN PATENT DOCUMENTS

127958A2	5/1984	European Pat. Off. .
0125139	11/1984	European Pat. Off. .
0184909	6/1986	European Pat. Off. .
0241309A3	4/1987	European Pat. Off. .
0278647	8/1988	European Pat. Off. .
0368209A1	6/1989	European Pat. Off. .
0390390A1	3/1990	European Pat. Off. .
55-012437A	1/1980	Japan .
83049821B	11/1983	Japan .
63-309848A	12/1988	Japan .
2-006737A	1/1990	Japan .
2-088960A	3/1990	Japan .
2099850A	4/1990	Japan .
830229A	5/1981	U.S.S.R. .

OTHER PUBLICATIONS

Anson et al., *J. of the Am. Chem. Soc.*, vol. 105, No. 15, (1983), pp. 4883–4890.

Anson et al., *J. of the Am. Chem. Soc.*, vol. 105, No. 5, (1983), pp. 1096–1106.

Foulds, et al., Immobilization of Glucose Onidase in Ferrocene-Modified Pyrrole Polymers, Anal. Chem., vol. 60, No. 22, 2473–8 (1988).

Hale, et al., A New Class of Amperometric Biosensor Incorporating a Polymeric Electron-Transfer Mediator, J. Am. Chem. Soc., vol. 111, No. 9, pp. 3482–3484 (1989).

Foulds, et al., Enzyme Entrapment in Electrically Conducting Polymers, J. Chem. Soc., Faraday Trans. 1, vol. 82, pp. 1259–1264 (1986).

Degani, et al., Electrical Communication Between Redox Centers of Glucose Oxidase and Electrodes via Electrostatically and Covalently Bond Redox Polymers, J. Am. Chem. Soc., vol. 111, pp. 2357–2358 (1989).

Bartlett, et al., Strategies for the Development of Amperometric Enzyme Electrodes, Biosensors, vol. 3, pp. 359–379 (1987/88).

Clark, et al., Electrode Systems for Continuous Monitoring in Cardiovascular Surgery, Ann N.Y. Acad. Sci., vol. 102, pp. 29–45 (1962).

Clark, et al., Long-term Stability of Electroenzymatic Glucose Sensors Implanted in Mice Trans. Am. Soc. Artif. Inten. Organs, vol. 34, pp. 259–265 (1988).

(List continued on next page.)

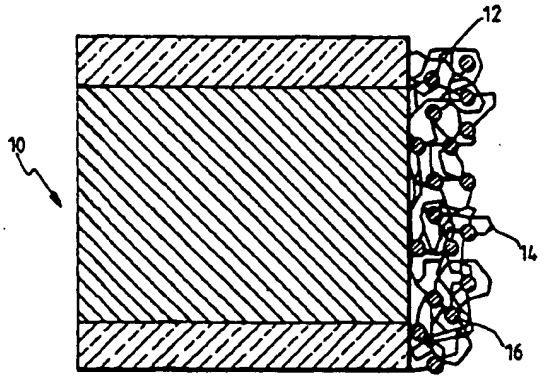
Primary Examiner—T. Tung

Attorney, Agent, or Firm—Pravel, Hewitt, Kimball & Krieger

[57] ABSTRACT

Enzyme electrodes having a surface coated with a film. The film is formed from materials in which a redox enzyme is covalently bonded to a three dimensional molecular structure. The molecular structure is of the class having multiple redox centers, for example, a crosslinked redox polymer.

18 Claims, 6 Drawing Sheets



US005813989A

United States Patent [19]

Saitoh et al.

[11] **Patent Number: 5,813,989**

[45] **Date of Patent: *Sep. 29, 1998**

[54] **DRIVING MENTAL CONDITION DETECTING APPARATUS**

[75] Inventors: **Satoshi Saitoh; Mitsuo Yasushi; Kazuhiro Akiyama; Masatoshi Yanagidaira**, all of Kawagoe, Japan

[73] Assignee: **Pioneer Electronic Corporation**, Tokyo, Japan

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: **559,273**

[22] Filed: **Nov. 15, 1995**

[30] **Foreign Application Priority Data**

Nov. 16, 1994 [JP] Japan 6-282219

[51] **Int. Cl.**[6] **A61B 5/00**; G08B 23/00

[52] **U.S. Cl.** **600/484**; 340/576

[58] **Field of Search** 128/671, 731, 128/733, 732, 898; 340/575, 576, 990, 995; 600/484, 544, 545, 546, 898

[56] **References Cited**

U.S. PATENT DOCUMENTS

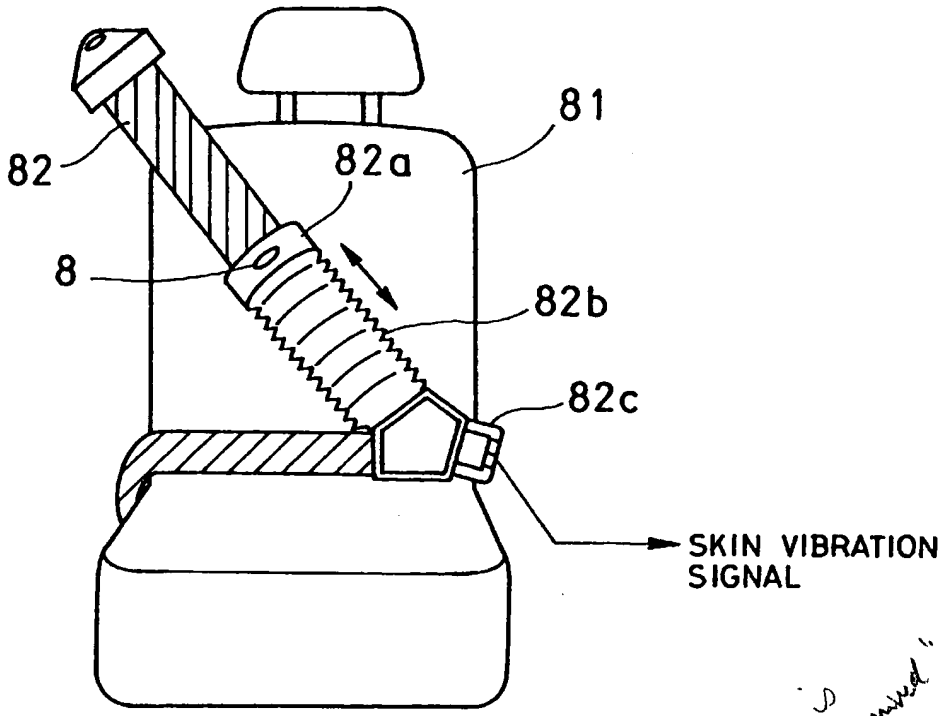
4,673,913	6/1987	Akita et al.	340/575 X
4,679,648	7/1987	Johansen	340/576 X
5,266,070	11/1993	Hagiwara et al.	600/27
5,485,385	1/1996	Mitsugi	340/990 X
5,488,353	1/1996	Kawakami et al.	340/575 X
5,513,110	4/1996	Fujita et al.	340/990 X

Primary Examiner—Jennifer Bahr
Assistant Examiner—Stephen Huang
Attorney, Agent, or Firm—Sughrue, Mion, Zinn, Macpeak & Seas, PLLC

[57] **ABSTRACT**

A driving mental condition detecting apparatus for detecting a deterioration state of driving mental conditions such as sleepiness, fatigue, and impatience occurring in a driver on the basis of physiological data detected from the driver and road travel data of a vehicle derived from a navigation system, thereby generating an alarm.

24 Claims, 18 Drawing Sheets



Correlation is "predetermined"

US005262035A

United States Patent [19]

Gregg et al.

[11] Patent Number: 5,262,035

[45] Date of Patent: Nov. 16, 1993

[54] ENZYME ELECTRODES

[75] Inventors: Brian A. Gregg; Adam Heller, both of Austin, Tex.

[73] Assignee: E. Heller and Company, Austin, Tex.

[21] Appl. No.: 389,226

[22] Filed: Aug. 2, 1989

[51] Int. Cl.[5] G01N 27/327

[52] U.S. Cl. 204/403; 204/153.12; 435/817; 436/518; 436/531; 436/806

[58] Field of Search 204/153.12, 403; 435/817; 436/806, 518, 531

[56] References Cited

U.S. PATENT DOCUMENTS

4,224,125	9/1980	Nakamura et al.	435/817
4,461,691	7/1984	Frank	204/290 R
4,476,003	10/1984	Frank et al.	204/290 R
4,524,114	6/1985	Samuels et al.	204/294
4,545,382	10/1985	Higgins et al.	204/403
4,619,754	10/1986	Niki et al.	204/290 R
4,655,885	4/1987	Hill et al.	204/290 R
4,711,245	12/1987	Higgins et al.	204/415
4,717,673	1/1988	Wrighton et al.	204/290 F
4,721,601	1/1988	Wrighton et al.	204/435
4,764,416	8/1988	Ueyama et al.	428/212
4,784,736	11/1988	Lonsdale et al.	204/157.15
4,894,339	1/1990	Hawazato et al.	435/817
4,917,800	4/1990	Lonsdale et al.	210/490
4,968,400	11/1990	Shimomura et al.	204/403
5,089,112	2/1992	Skotheim et al.	204/153.12

FOREIGN PATENT DOCUMENTS

127958A2	5/1984	European Pat. Off. .
0125139	11/1984	European Pat. Off. .
0184909	6/1986	European Pat. Off. .
0241309A3	4/1987	European Pat. Off. .
0278647	8/1988	European Pat. Off. .
0368209A1	6/1989	European Pat. Off. .
0390390A1	3/1990	European Pat. Off. .
55-012437A	1/1980	Japan .
83049821B	11/1983	Japan .
63-309848A	12/1988	Japan .
2-006737A	1/1990	Japan .
2-088960A	3/1990	Japan .
2099850A	4/1990	Japan .
830229A	5/1981	U.S.S.R. .

OTHER PUBLICATIONS

Anson et al., *J. of the Am. Chem. Soc.*, vol. 105, No. 15, (1983), pp. 4883–4890.

Anson et al., *J. of the Am. Chem. Soc.*, vol. 105, No. 5, (1983), pp. 1096–1106.

Foulds, et al., Immobilization of Glucose Onidase in Ferrocene-Modified Pyrrole Polymers, Anal. Chem., vol. 60, No. 22, 2473–8 (1988).

Hale, et al., A New Class of Amperometric Biosensor Incorporating a Polymeric Electron-Transfer Mediator, J. Am. Chem. Soc., vol. 111, No. 9, pp. 3482–3484 (1989).

Foulds, et al., Enzyme Entrapment in Electrically Conducting Polymers, J. Chem. Soc., Faraday Trans. 1, vol. 82, pp. 1259–1264 (1986).

Degani, et al., Electrical Communication Between Redox Centers of Glucose Oxidase and Electrodes via Electrostatically and Covalently Bond Redox Polymers, J. Am. Chem. Soc., vol. 111, pp. 2357–2358 (1989).

Bartlett, et al., Strategies for the Development of Amperometric Enzyme Electrodes, Biosensors, vol. 3, pp. 359–379 (1987/88).

Clark, et al., Electrode Systems for Continuous Monitoring in Cardiovascular Surgery, Ann N.Y. Acad. Sci., vol. 102, pp. 29–45 (1962).

Clark, et al., Long-term Stability of Electroenzymatic Glucose Sensors Implanted in Mice Trans. Am. Soc. Artif. Inten. Organs, vol. 34, pp. 259–265 (1988).

(List continued on next page.)

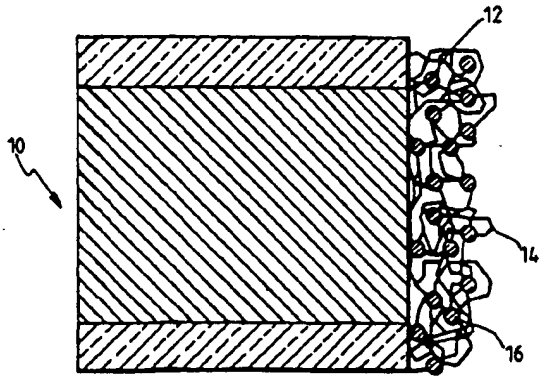
Primary Examiner—T. Tung

Attorney, Agent, or Firm—Pravel, Hewitt, Kimball & Krieger

[57] ABSTRACT

Enzyme electrodes having a surface coated with a film. The film is formed from materials in which a redox enzyme is covalently bonded to a three dimensional molecular structure. The molecular structure is of the class having multiple redox centers, for example, a crosslinked redox polymer.

18 Claims, 6 Drawing Sheets



SPL decrease curves below the set point; gradient ranges A, B, and C. The decrease curve of SPL within gradient range A results from diurnal variation of SPL or acclimatization to driving circumstance. The decrease curve of SPL within the gradient range C is caused by an external stimulus. Therefore, decrease of SPL can be detected by a gradient within the gradient range B. The arousal level judging apparatus of the third embodiment detects decrease of arousal level by the gradient of SPL curve below the set point which is determined by the reference value. The reference value is obtained from SPL just after occurrence of SPR.

The operation of the CPU 3*b* of the third embodiment will now be described with reference to FIGS. 13A and 13B.

The CPU 3*b* of the arousal level judging apparatus of the third embodiment operates, as shown in FIG. 13A, by first determining a set point from the reference value. Then, if SPL decreases to the set point and further SPL decreases at a greater ratio of decrease ratio than a predetermined ratio, i.e., the gradient of the SPL curve becomes steeper than before after the set point SP, the CPU 3*b* sends a doze signal to the buzzer 4 and air-conditioning controller 5 to arouse the driver.

More specifically, the CPU 3*b* starts an operation with power being supplied, then, entering the operation flow shown in FIG. 13A at step 400. In step 400, an initialization is made for clearing a RAM and setting flags as well as set initial data for controlling, such as a predetermined value S1 for judging SPR occurrence through SPL variation and a predetermined value S2 for judging SPL decrease due to an arousal level decrease. Next, in step 410, the CPU 3*b* reads a value of SPL from A/D converter 3*a* and stores the SPL k (k indicates the number of times of processing at step 410). In the succeeding step 420, the CPU 3*b* calculates gradient of SPL change $-V/t$ for detection of SPR. In actuality, the CPU 3*b* subtracts the value of SPL $k-1$ from that SPL k and waits for time interval where t (t is unit time). This shows gradient of SPL change. A decision is then made, in step 430, as to whether the resultant gradient is smaller than a predetermined value T for detection of SPR, i.e., detecting a pulse of SPR, i.e., detecting the gradient of SPL indicated by $-V1$ and t1 in FIG. 4, which is smaller, or steeper than the gradient $-V2/t2$. If the resultant gradient is smaller than the predetermined value then this indicates that the present change of SPL is made by SPR, in which case step 452 is executed. At the first processing of step 430, the CPU 3*b* decides SPL change of SPR does not occur, then processing proceeds around the loop of steps 440, 450, 410, 420, and 430, until the SPL change of SPR is detected in step 430. If SPR is detected in step 430, process proceeds to step 452. In step 452 SPR flag is set, then processing proceeds to step 410 through step 450. At the first, SPR is not detected in step 430, then processing proceeds to step 440.

In step 440, a decision is made as to whether convergence of an SPL change of SPR is detected. If convergence of the SPL change of SPR is detected, SPR convergence flag is set in step 440. At the first operation of step 440, the CPU 3*b* determines that SPR has been not converged, then processing returns to step 410 through step 450.

FIG. 13B shows the detailed steps of step 440 of FIG. 13A. The process of step 440 begins at step 440*b*. A determination is made in the following step 446, as to whether "convergence flag" has been set. If the "convergence flag" has been set, processing progresses to the outlet of the flow diagram, i.e., "YES" terminal. If the convergence flag is not set, a decision is made in the step 448, as to whether SPR flag has been set. If SPR flag has been set, the processing proceeds to step 441. A decision is made in step 441, as to whether gradient of SPL change is greater than a positive predetermined value R1. If a gradient is greater than the positive predetermined value R1, processing proceeds to step 443. At the first operation of step 441, a gradient is lower than the value R1 then this indicates that the SPL change enters negative slope change as shown by stage 1 in FIG. 8D. Then, a decision is made in step 442 as to whether a gradient of SPL is nearly equal to zero, then this indicates the present SPL to reach convergence of SPR pulse. At the first operation of step 442, the CPU 3*b* judges the gradient not equal nearly to zero then processing progresses to "NO" of step 440, i.e., to step 110. When SPL change enters the stage 3, the CPU 3*b* judges SPL change positive then setting a plus flag in step 441. In convergence stage of SPR, i.e., stage 4, processing proceeds through step 441, to step 442. The CPU 3*b* judges a gradient nearly equal to zero, and then the process progresses to step 444. A decision is made as to whether the plus flag has been set, then this indicates that SPL change has passed the bottom stage of SPL change of SPR. Therefore, if SPL change is at the bottom stage of SPR the operation progresses to "NO" of step 440. In convergence stage of SPR, processing progresses to step 445. Then, the CPU 3*b* detects convergence of SPL change of SPR. In the following step 445, the CPU 3*b* resets the plus and SPR flags. In the successive step 447, "convergence flag" is set. Then, processing proceeds to "YES" of step 440.

In FIG. 13A, processing progresses from the "YES" terminal to step 460. A combination of steps 460 and 470 provides a counter for processing cycle count. At first processing of steps 460 and 470, processing proceeds to step 480. In step 480, the CPU 3*b* stores values of SPL and calculates arithmetic mean as well as, calculating the set point SP from the resultant arithmetic mean, then processing progresses around the loop of steps 490, 410, 430, 440, 460, 470, and 480 until the loop count reaches 3 in counting steps 460 and 470 in order to confirm that SPR convergence status continues for a predetermined interval. However, if another SPR occurs during process of the loop of steps 490, 410, 430, 440, 460, 470, and 480, processing branches off at step 430 and proceeds to steps 450, then, the stored SPL data and convergence, plus, and flags are cleared and the CPU 3*b* begins storing new SPL data.

When the counting has finished, processing progresses to step 500. In step 500, CPU 3*b* compares a sampled SPL value with the set point value SP. If SPL is higher than the set point value SP, process return to step 410. If SPL is lower than the set point (stage 7), SPL change V/t is compared with a predetermined value S2 in step 510. In step 500, if the value of SPL gradient $-V/t$ is larger than the predetermined value S2, a drowsiness signal is outputted in step 520 (stage 8). If SPL gradient $-V/t$ is smaller than the predetermined value S2, i.e., SPL gradient is steeper than that determined by S2, this decrease of SPL can be determined as an SPL decrease of arousal level decrease because in step 430 this decrease was determined an SPL change other than that of SPR, i.e., of gradient range A. Then, the CPU 3*b* send the drowsiness signal

OTHER PUBLICATIONS

Cass, et al., Ferricinium as an Electron Acceptor for Oxido-Reductases, J. Electroanal. Chem., vol. 190, pp. 117-127 (1985).

Albery, et al., Amperometric Enzyme Electrodes, Phil. Trans. R. Soc. Lond., vol. B 316, pp. 107-119 (1987).

Scheller, et al., Enzyme Electrodes and Their Application, Phil. Trans. R. Soc. Lond, vol. B 316, pp. 85-94 (1987).

Pollak, et al., Enzyme Immobilization by Condensation Copolymerization into Cross-Linked Polyacrylamide Gels, J. Am. Chem. Soc., vol. 102, No. 20, pp. 6324-6336 (1980).

Castner, et al. Mass Transport and Reaction Kinetic Parameters Determined Electrochemically for Immobilized Glucose Oxidase, Biochemistry, vol. 23, No. 10, pp. 2203-2210 (1984).

Gregg et al., "Cross-Linked Redox Gels Containing Glucose Oxidase for Amperometric Biosensor Applications", 1990, pp. 258-263.

Coughlan, "Concepts and Approahces to the Understanding of Electron Transfer Processes in Enzymes Containing Multiple Redox Centers", 1980, Chapter 5, *Molybdenum-Containing Enzymes*, pp. 187-220.

Bartlett, et al., "Covalent Binding of Electron Relays to Glucose Oxidase," J. Chem. Soc., Chem. Commun., pp. 1693-2004 (1987).

Umana, U.S. Army Research Office Report No. ARO 23106.3-LS entitled "Protein-Modified Electrochemically Active Biomaterial Surface", dated Dec. 1988.

Samuels et al., "An Electrode-Supported Oxidation Catalyst Based on Ruthenium(IV). pH 'Encapsulation' in a Polymer Film," J. Am. Chem Soc., vol. 103, pp. 307-312 (1981).

Denisevich et al., "Unidirectional Current Flow and Charge State Trapping at Redox Polymer Interfaces on Bilayer Electrodes: Principles, Experimental Demonstration, and Theory," J. Am. Chem. Soc., vol. 103, pp. 4727-4737 (1981).

Abruna et al., "Rectifying Interfaces Using Two-Layer Films of Electrochemically Polymerized Vinylpyridine and Vinylbipyridine Complexes of Ruthenium and Iron on Electrodes," J. Am. Chem. Soc., vol. 103, pp. 1-5 (1981).

Ellis et al., "Selectivity and Directed Charge Transfer Through an Electroactive Metallopolymer Film," J. Am. Chem. Soc., vol. 103, pp. 7480-7483 (1981).

Degani, Y. and Heller, A., "Direct Electrical Communication Between Chemically Modified Enzymes and Metal Electrodes. 1. Electron Transfer from Glucose Oxidase to Metal Electrodes via Electron Relays, Bound Covalently to the Enzyme," Journal of Physical Chemistry, vol. 91, pp. 1285-1289 (1987).

Degani, Y. and Heller, A., "Direct Electrical Communication Between Chemically Modified Enzymes and Metal Electrodes. 2. Methods for Bonding Electron-Transfer Relays to Glucose Oxidase and D-Amino-Acid Oxidase," JACS, vol. 110, pp. 2615-2620 (1988).

proceeds to steps 140 and 160, then, the stored SPL data and convergence, plus, and zero flags are cleared and the CPU 3*b* begins storing new SPL data.

When the counting has finished at step 210, the CPU 3*b* averages five stored SPL values and stores the resultant mean data as a reference value in step 220. In the succeeding step 230, a reference flag is set and convergence and SPR flags are reset, then process returns to step 110. Therefore, in this status, the CPU 3*b* is permitted to execute the calculation process of steps 240, 250, 260, and 270 in step 170. Then, processing proceeds to steps 250 and 260. If SPL decrease ratio is lower than a predetermined value X, then the doze signal is sent to the buzzer 4 and air-condition controller 5 in step 270. If not so, processing returns to step 110.

Then, the buzzer 4 alarms and the air-condition controller 5 operates the blower 6*b*, compressor 6*d* as well as opens the by-pass damper 6*g* in order to supply a cool air toward the driver in response to the drowsiness signal. Therefore, the driver is notified his arousal level has decreased and is aroused by the cool air.

As stated hereinabove, the arousal level judging apparatus of the first embodiment stores the value of SPL just after SPR occurrence as a reference value for determining SPL decrease ratio, as well as the reference is renewed at every occurrence of SPR. This makes it possible to judge arousal level of the driver having been decreased to drowsiness level, eliminating effects of diurnal variation, acclimatization, individual difference. Therefore, the arousal level judging apparatus of the first embodiment provides accurate detection of arousal levels.

Hereinbelow will be described the second embodiment.

An arousal level judging apparatus of the second embodiment has a similar structure to the first embodiment However, the second embodiment operates according to the program of FIG. 8A. Also input circuit 8 shown in FIG. 7 is provided which receives and sends data to the CPU 3*b*. Therefore, a detailed description of the circuit arrangement of the second embodiment is omitted.

FIG. 9A represents SPL diurnal variations of high arousal level and low arousal level. FIG. 9A is obtained through an experiment. The experiment was made as follows:

In an ordinary room, SPL of a subject is measured by using detection electrodes attached to his body. The subject is given high and low arousal states alternately at a predetermined interval, for example, five or twenty minites. The high arousal state is made by conversation. The low arousal state is made by leaving the subject at a rest-and-closing eye state. The experiment was done from about 10 o'clock to 22 o'clock. Next, obtained SPLs of high arousal and low arousal levels are plotted respectively, as shown in FIG. 9A. Here, low arousal state means a rest-and-closing eyes state but not sleeping state. This means that rest-and-closing eye states is dangerous when driving a car. FIG. 9B shows the difference between high arousal level and low arousal level.

In FIG. 9A, SPL which has been obtained from a human being in high arousal state varies as the passage of time within a day, as shown by an upper curve. As described above, in the first embodiment, the arousal level judging apparatus calculates SPL decrease ratio as defined by Eq. (1) by using absolute values of SPL. This means that when the reference value is high, decrease amount of SPL is large and when the reference value is low, decrease amount of SPL is small. In other words, in the method according to the first embodiment it is assumed that decrease amount of SPL representing the same arousal level decrease is proportional to the reference value. However, experiments show that SPL difference between high and low arousal levels, for each driver is substantially constant, as shown in FIG. 9B. Therefore, if degree of arousal level decrease are the same between high and low arusal states, SPL decrease amounts should also be changed in accordance with the varying reference value. Then, the predetermined value X should be changed in accordance with reference values. In the second embodiment, the decrease ratio is calculated on the basis of the diference D which is a mean value, as shown in FIG. 9B. Experiments also show that the difference D is different from one another between drivers. Therefore, in the second embodiment of the present invention, input means 8 is provided which receives and sends individual data to the CPU 3*b*. The CPU 3*b* calculate SPL decrease ratio by the following equation:

$$\text{SPL decrease ratio} = (\text{reference value} - \text{present SPL})/\text{SPL difference between high and low arousal levels } (D) \quad (2)$$

In the second embodiment of the present invention, the CPU 3*b* is operated by the program shown in the flow diagram of FIG. 8A and SPL difference D is inputted to CPU 3*b* in step 102, as shown in FIG. 8C, which is located just after step 100 and the equation (2) is used in step 250 in FIG. 8A.

In this way, the arousal level judging apparatus of the second embodiment can judge the arousal level of a driver more accurate than the first embodiment because arousal level is determined on the basis of SPL difference D for normalization in the second embodiment, whereas, in the first embodiment, arousal level is determined on the basis of SPL difference between the reference value and zero level. In addition, difference D can be inputted individually, so that arousal level can be judged more accurate than the arousal level judging apparatus of the first embodiment. Moreover, difference D may also be preset in the CPU 3*b*. Further, arousal level decrease can also be detected in steps 250, and 260 by comparing SPL decrease which is obtained by substracting the present value of SPL from the reference value with a predetermined value.

Hereinbelow will be described the third embodiment of an arousal level judging apparatus of the present invention.

The third embodiment of the arousal level judging apparatus has the same structure as that of the first embodiment but uses the program of FIG. 13A–13B for CPU 3*b* as described later. Therefore, a detailed description of the circuit arrangement of the third embodiment is omitted.

FIG. 11 shows a curve of SPL variation when a driver falls asleep. In the early stage of driving a car, a change of SPL due to SPR occurs. Then, SPL decreases from a reference value RV to a set point SP with the time passing because of acclimatization. The SPL variation curve below the set point SP has a larger gradient than that from the reference value to the set point. This SPL variation curve shows that the driver falls almost asleep after time t3. Therefore, a decrease of arousal level can be detected by the gradient of SPL curve below the set point. FIG. 12 shows three types of

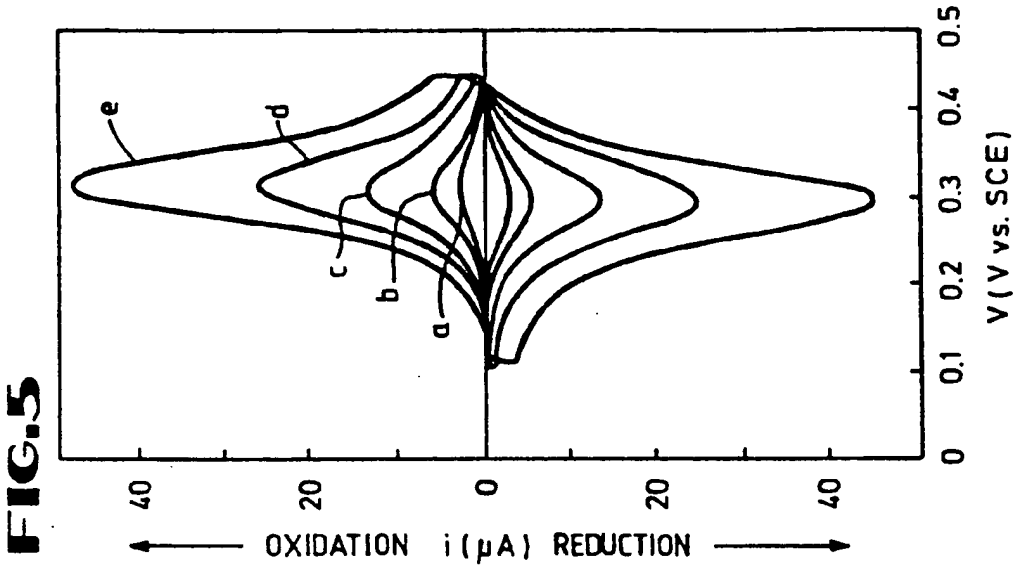

e
d
c
b
a
0
0.1
0.2
0.3
0.4
0.5
V (V vs. SCE)
40
20
0
20
40
OXIDATION i (μA) REDUCTION

FIG.5

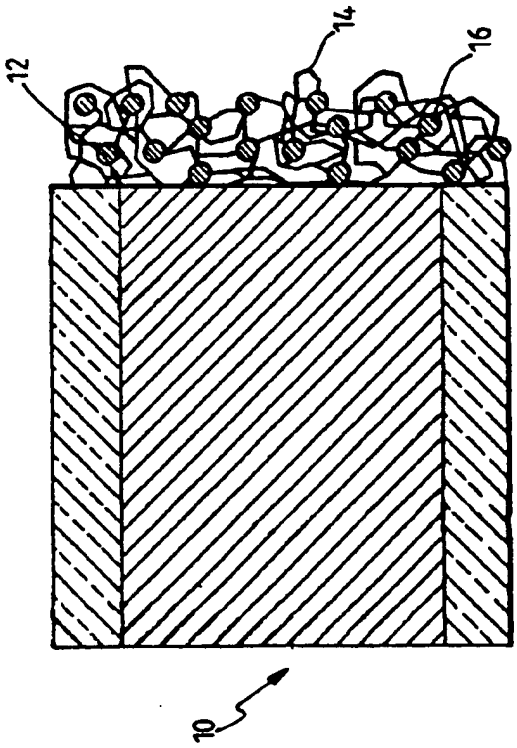

12
14
16
10

FIG.1

CPU 3*b* sends a drowsiness signal to the buzzer 4 and the air condition controller 5. The buzzer 4 alarms in response to the drowsiness signal and gives a driver a warning in order to prevent the driver from sleeping. The air condition controller 5 also operates the blower 6*b*, the compressor 6*d*, by-pass servo motor 6*h* which opens the by-pass damper 6*g*, in response to the drowsiness signal, thereby, blowing a cool air toward the driver's face. This causes the driver to be aroused.

Hereinbelow will be described general operation of the first embodiment.

In FIG. 3, SPLs just after SPR occurrences respectively returns to nearly the same level. When SPR occurs in a drive stage of about one hour passed, the decreased SPL returns to the level B which is nearly the same as level A from which SPL begins to decrease. Therefore, an SPL just after occurrence of a SPR shows SPL of the driver at a high arousal level. The arousal level judging apparatus of the first embodiment stores the value of SPL just after a SPR occurrence as a reference value for determining SPL decrease ratio, as well as the reference is renewed at every occurrence of SPR. This makes it possible to judge the arousal level decreased to drowsiness level, eliminating effects of diurnal variation, acclimatization, and individual differences. In the first embodiment of the arousal judging apparatus, a decrease ratio of SPL is obtained by the following equation:

$$\text{decrease ratio of } SPL = \text{present } SPL/SPL \text{ just after } SPR \text{ occurrence} \quad (1)$$

In an experiment, such as shown in FIG. 3 made by the inventors, subjects of car drivers said that they had felt drowsiness when the decrease ratio of SPL decreased to a range from 0.6 to 0.8. Therefore, the arousal level judging apparatus is so designed as to judge a driver drowsiness when the decrease ratio of SPL decrease to this range.

The above-mentioned operation can be performed by a CPU 3*b*. Hereinbelow will be described operation of the CPU 3*b* of the first embodiment with reference to FIG. 8A of a flow chart.

The CPU 3*b* starts an operation with turning power on, then, entering the operation flow shown in FIG. 8A at step 100. In step 100, an initialization is made for clearing a RAM and setting flags, etc. Next, in step 110, the CPU 3*b* reads a value of SPL from A/D converter 3*a* and stores the value of SPL k (k indicates the number of times processing at step 110). In the succeeding step 120, the CPU 3*b* calculates gradient of SPL change $-V/t$ (t is unit time interval) for detection of SPR occurrence. In actually, the CPU 3*b* subtracts the value of SPL k−1 from that of SPL k and waits for time interval t. This shows gradient of SPL change. Practically, processing time interval should be considered. However, because process speed is extremely high and processing time interval is not so long comparing with time interval t, thus, process time interval can be omitted. A decision is then made, in step 130, as to whether the resultant gradient is lower than a predetermined value T, i.e., threshold level T, for detecting a pulse of SPR, i.e., detecting the gradient of SPL, as indicated by $-V1$ and t1 in FIG. 4 which is lower or steeper than the gradient $-V2/t2$. If the resultant gradient is lower than the predetermined value T then this indicates that the present change of SPL is made by SPR, in which case step 140 is executed. At the first processing of step 130, the CPU 3*b* decides SPL change of SPR does not occur, then processing proceeds around the loop of steps 170, 240, 270, and 110, until the SPL change of SPR is detected in step 130, i.e., SPL change enters stage 1 shown in FIG. 8D. If SPR is detected in step 130, process proceeds through step 150, in which SPR flag is set, to step 170 in which detects SPR flag. If SPR flag is detected, then process proceeds through step 180 to step 190. A decision is made in step 190 as to whether convergence of SPR pulse is detected. If convergence of SPR pulse is detected, i.e., this means SPL change enters stage 4 shown in FIG. 8D, SPR convergence flag is set in step 200. Then, the value of SPL n is stored and process proceed to step 110 through step 210. At the first operation of step 190, the CPU 3*b* decides that SPR has not converged, then processing returns to step 110.

Now, the SPR convergence flag is set at every detection of SPR and is reset at every processing of step 230. If the SPR convergence flag is set in step 200, i.e., this means SPL enters stage 4, 5, it forbids the CPU 3*b* to proceed to the loop of steps 240–270 until a reference value is finally determined in step 220.

The SPR convergence flag forbids the CPU 3*b* to read the value of SPL during stage 1, 2, and 3. When it is detected that gradients of SPL change, i.e., positive and negative values of V/t, are in the predetermined ranges, the SPR convergence flag is set in step 200. The flag permits the CPU 3*b* to read SPL in for determination of the reference value.

FIG. 8B shows the process of step 190 more clearly. The process of step 190 begins at step 190*b*. A decision is made in the successive step 191, as to whether the gradient of SPL change is greater than a positive predetermined value R1. If a gradient is larger than the positive predetermined value R1, i.e., this means SPL change enters stage 3, process proceeds to step 193. At the first operation of step 191, a gradient is smaller than the predetermined value R1, then this indicates that the SPL change enters stage 1 shown in FIG. 8D. Then, a decision is made in step 192, as to whether a gradient of SPL is nearly equal to zero, then this indicates that the present SPL reaches convergence of SPR pulse. At the first operation of step 192, the CPU 3*b* judges the gradient not equal nearly to zero, then process progresses to "NO" of step 190, i.e., to step 110. If SPL change enters stage 3 shown in FIG. 8D, the CPU 3*b* judges SPL gradient positive then setting a plus flag in step 193. In convergence stage of SPR, i.e., stage 4, process progresses through step 191, to step 192. The CPU 3*b* judges a gradient nearly equal to zero, then process progresses to step 194. A decision is made as to whether the plus flag has been set, then this indicates that SPL change has passed the bottom of SPL change of SPR. Therefore, if SPL change reaches the bottom stage of SPR the operation progresses to "NO" of step 190. In convergence stage of SPR, process progresses to step 195. Then, the CPU 3*b* detects convergence of SPL change of SPR. In the successive step 195, the CPU 3*b* resets the plus flag. Then, process progresses to "YES" of step 190.

Next, the process progresses to step 200 in FIG. 8A. The CPU 3*b* sets the convergence flag. In step 205, the CPU 3*b* stores a value of SPL, then the processes progresses around the loop of steps 205, 210, 110, 170, 180, 205, and 210 until the loop count reaches 5 in counting steps 205 and 210. However, if another SPR occurs during process of the loop of steps 205, 210, 110, 170, 180, 205, and 210, process branches off at step 130 and

FIG.2A

POLYMER A

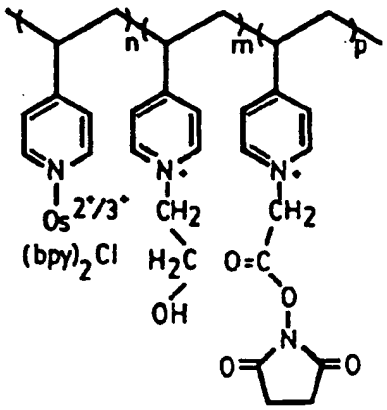
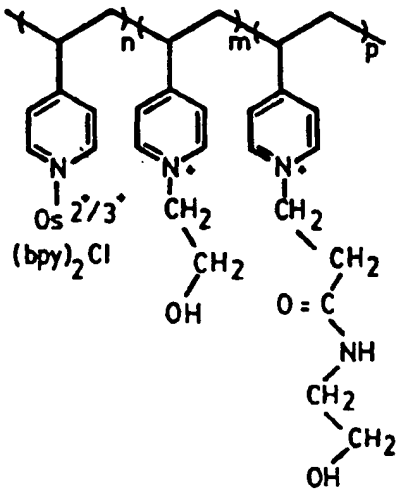
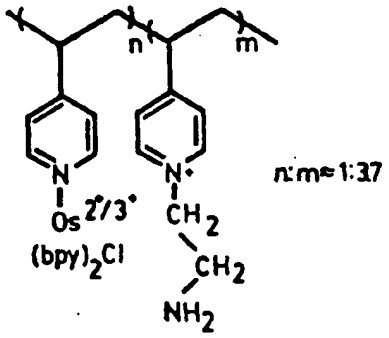


FIG.2B

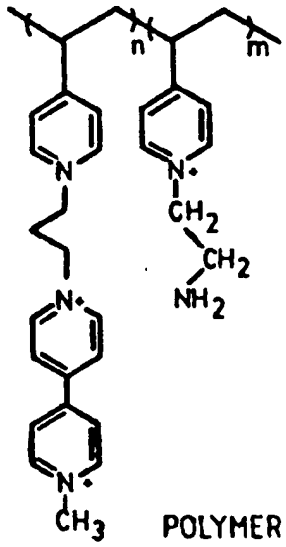
POLYMER B





POLYMER C

FIG.2C



POLYMER D

FIG.2D

below said set point value is grater than a second predetermined value.

The present invention also defines a method for accomplishing the above discussed objectives.

BRIEF DESCRIPTION OF THE DRAWINGS

The object and features of the present invention will become more readily apparent from the following detailed description of the preferred embodiments taken in conjunction with the accompanying drawings in which:

FIG. 1 is a general block diagram of the present invention;

FIG. 2 is a drawing showing a SPL variation within a day;

FIG. 3 is a drawing showing a typical SPL variation curve during driving an automobile;

FIG. 4 is a drawing showing a SPL variation curve with SPR and another SPL variation;

FIG. 5 is a histogram of SPL gradients;

FIG. 6 is a block diagram of a first embodiment;

FIG. 7 is a partial block diagram of a second embodiment showing a portion of FIG. 6;

FIGS. 8A and 8B are flow charts of the first embodiment;

FIG. 8C is an additional flow chart of a second embodiment which is executed after step 100 of FIG. 8A;

FIG. 8D is a drawing showing an SPL variation curve divided in nine stages;

FIG. 9A is a drawing showing a SPL variation within a day;

FIG. 9B is a drawing showing a difference variation of SPL between high and low arousal states;

FIG. 10 is a block diagram of another preferred embodiment;

FIGS. 11 and 12 are drawings showing SPL variation curves; and

FIGS. 13A and 13B are flowcharts of a third embodiment.

The same or corresponding elements and parts are designated at like reference numerals throughout the drawings.

DETAILED DESCRIPTION OF THE INVENTION

Referring to FIGS. 1 to 6, 8A, 8B and 8D, a first embodiment of the present invention will be described.

FIG. 1 is a functional block diagram showing basic functions performed by the arousal level judging apparatus according to the present invention which are common to all the embodiments. In FIG. 1, an arousal level judging apparatus comprises a skin potential detector 1 which detects skin potential level (SPL) of human body, a SPR detector 9 for detecting pulse type of SPL variation, i.e., for detecting skin potential response (SPR) occurrence and convergence of SPR, responsive to the skin potential detector 1, and for storing SPL value, and an arousal level comparator 10 for comparing stored SPL with current SPL to detect arousal level decrease. Here, convergence of the SPL curve means that SPL change due to SPR reaches non-pulse like portion, i.e., stage 4 shown in FIG. 8D, through stages 1 to 3.

The arousal level judging apparatus of the first embodiment mounted in an automobile detects a decrease of arousal level of an automobile driver and alarms the driver by an alarm bell and wakes the driver up.

Hereinbelow will be described a general operation of the arousal level judging apparatus.

FIG. 3 shows variation of SPL during driving an automobile. In early stage of driving a car, SPL stays at a high level and several SPRs occur with environmental changes, i.e., stimuli such as conversation and passing another car ahead.

However, SPL begins to decrease gradually after about one hour has passed because of acclimatization to driving of the car and driving environment. In this stage, the frequency of SPR occurrences decrease, then, SPL decreases with sawtooth variations. This state continues until a large amount of stimuli, such as conversation, is applied to the driver. In other words, occurrence of SPR indicates the driver at a high arousal level.

The first embodiment has made for judging decrease in arousal level, utilizing the reference obtained from convergence of SPL curve due to SPR and decrease ratio of SPL to the reference.

Hereinbelow will be described detection of SPR where SPL change of SPR should be distinguished from SPL diurnal change and decrease of the arousal level.

FIG. 4 shows a SPL variation curve with SPR and another SPL variations. This SPL variation curve is experimentally obtained and is schematically illustrated. FIG. 5 is a histogram showing frequencies of sampled data of SPL with respect to gradient of SPL curves. The occurrence frequency of arousal level decrease type is indicated by hatched bars. The scale of the transverse is logarithm.

FIG. 4 shows that SPL gradient of SPR ($-V_1/t_1$) is larger than that of diurnal change or decrease in arousal level ($-V_2/t_2$). FIG. 5 shows the difference in occurrence inclination between SPL decrease of SPR and that of other causes with respect to V/t. Therefore, occurrence of SPR can be detected by comparing $-V/t$ of SPL variation with a threshold level T. The absolute value of SPR gradient is larger than that of other causes.

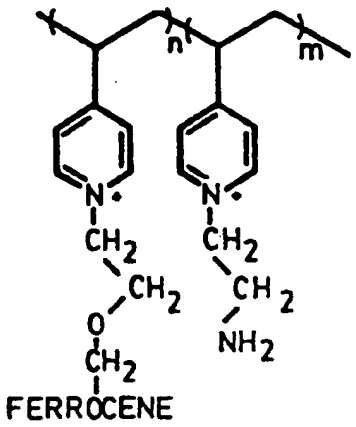
FIG. 6 shows a block diagram of an arousal level judging apparatus according to the present invention.

In FIG. 6, SPL detection electrodes 1*a*, 1*b* are attached to the forearm of a driver and to the thenar area of the palm with paste, etc. The electrodes 1*a*, 1*b* are skin electrodes made of Ag-AgCl, etc. A detection circuit 2 differentially amplifies SPL signals from the electrodes 1*a*, 1*b* of the forearm and palm up to a several-volts level and outputs positive SPL signal (hereinafter referred to as SPL signal). The arousal level calculator 3 has A/D converter 3*a* which converts an analog SPL signal into a digital signal and a micro-computer (hereinafter referred to as CPU) 3*b* which executes the program mentioned later. A buzzer 4 and air condition controller 5 operate in response to signals from the CPU 3*b*. A car air conditioner 6 comprises a control damper 6*a* for selecting fresh or recirculated air, blower 6*b*, evaporator 6*c*, compressor 6*d*, heater 6*e*, air mixing damper 6*f*, by-pass damper 6*g*, by-pass servo motor 6*h*, by-pass air vent 6*i*, etc. The air vent 6*i* is directed toward the face of the driver.

Hereinbelow will be described structure of the first embodiment of the arousal level judging apparatus with respect to FIG. 6.

In FIG. 6, the operation of ignition key (unshown) causes the arousal level judging apparatus to be supplied with power and this causes CPU 3*b* to start executing a program of arousal level judgement. When CPU 3*b* judges the driver's arousal level to be low,

FIG.2E
POLYMER E



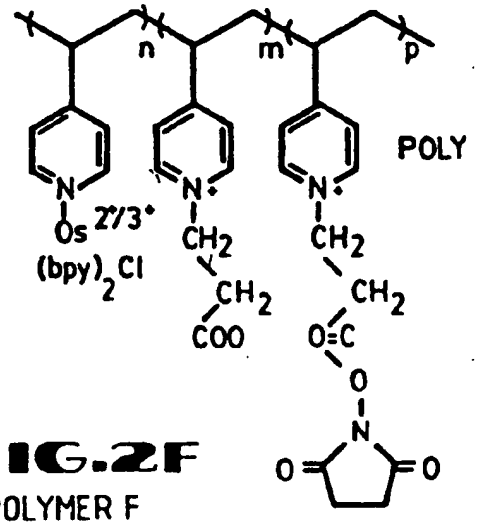


FIG.2F
POLYMER F

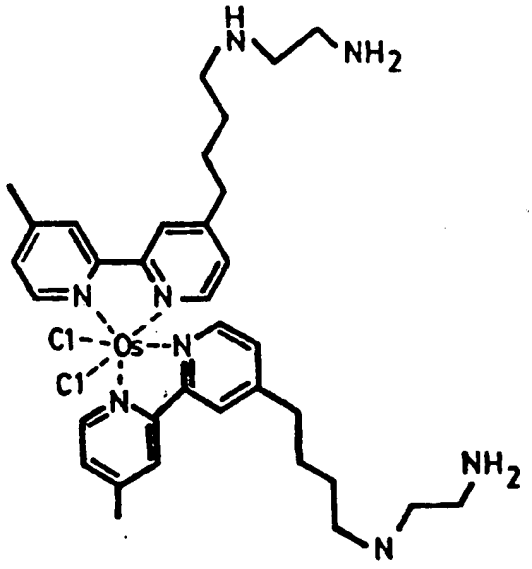


FIG.2G
POLYMER G

AROUSAL LEVEL JUDGING APPARATUS AND METHOD

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to an arousal level judging apparatus used for detecting an arousal level of an operator or a patient and more particularly, the invention relates to apparatus for detecting drowsiness during driving an automobile, which is applicable to a doze alarm system.

2. Description of the Prior Art

The main types of arousal level judging apparatus used for detecting the driver falling asleep which are known comprise; detection means having electrodes attached to a human body for detecting a skin potential level (hereinafter referred to as SPL) and a comparator for comparing the signal from the detection means with the predetermined value. Such technique has been described in Japanese patent application provisional publication No. 60-139539.

Generally, electrophysiological signals are known to have individual differences and variations within a day, i.e., diurnal variation. SPL also varies with diurnal variation, as shown in FIG. 2.

In the above-mentioned Prior art, in order to obtain a reference value to determine decrease ratio of SPL for judging a person falling asleep, a pre-experiment of sleep should be made before such detection of falling asleep is made actually because the reference value is required for compensation for the individual difference and diurnal variation.

However, an arousal level judging apparatus using the above-mentioned technique lacks ease in operation because such apparatus requires a user, for example, a car driver, to be subjected a pre-experiment of sleep. Although a compensation for general individual differences can be obtained through the above-mentioned pre-experiment, this compensation is ineffective for diurnal SPL variation because the reference to SPL will change with the passage of time.

Therefore, in the Prior art arousal level judging apparatus, there are drawbacks that it is difficult to obtain the reference to SPL, and that the reference should be determined at every predetermined intervals.

SUMMARY OF THE INVENTION

The present invention has been developed in order to remove the above-described drawbacks inherent to the conventional arousal level judging apparatus.

It is, therefore, an object of the present invention to provide a new and useful arousal level judging apparatus which is capable of detecting arousal level decrease without such pre-experiment and to renew the reference with the passage of time.

According to a feature of the present invention, the reference of SPL is obtained just after a pulse-like change of SPL, i.e., skin level potential response (hereinafter referred to as SPR), is substantially converged. The convergence of SPR is detected by SPR convergence detection means. The reference is stored by SPL storing means responsive to SPR convergence detection means. SPR occurs in accordance with an environmental change received by the user, i.e., subject. The occurrence of SPR reflects high arousal level of the user. This indicates that SPL of SPR-occurrence state can be adopted as a reference for detecting decrease in arousal level. This is done by storing the value of SPL. Then, SPL is compared with the reference value by a comparator for detecting a decrease in arousal level. SPL is detected by SPL detection means.

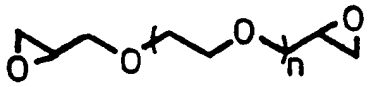
In accordance with the present invention there is provided an arousal level judging apparatus having SPL detection means, SPR convergence detection means, storing means for storing a reference value, SPL decrease ratio detection means which detects decrease of SPL by comparing measured SPL with a value obtained by multiplying the reference value with a predetermined value, and a comparator for further comparing decrease ratio with another predetermined value for detecting higher degree of SPL decrease than that detected by the SPL decrease ratio detection means, responsive to SPL decrease ratio detection means.

In accordance with the present invention there is further provided an arousal level judging apparatus comprising: skin potential level detection means for detecting skin potential level of a human body; level change detection means (110, 120) for detecting the degree of change in the level of an output signal of said skin potential level detection means over a predetermined interval; a comparing means (130) for comparing said degree from said level change detection means with a first predetermined value to detect the presence of a pulse-like change in said skin potential level; convergence detection means (190) responsive to an output signal from said comparing means (130) for detecting a non-pulse like portion in said skin potential level by analyzing succesive change of gradient determined by said degree from said level change detection means; storing means (220) for storing said skin potential level as a reference value when non-pulse like portion is detected by said convergence detection means; and arousal level detecting means (250, 260) responsive to said skin potential level from said skin potential level detection means and to said reference value from said storing means for determining arousal state and non-arousal state of said human body using the relation ship between said skin potential level and said reference value.

In accordance with the present invention there is further provided an arousal level judging apparatus comprising: skin potential level detection means for detecting skin potential level of a human body; level change detection means (410, 420) for detecting the degree of change in the level of an output signal of said skin potential level detection means over a predetermined interval; a comparing means (430) for comparing said degree from said level change detection means with a first predetermined value to detect the presence of a pulse-like change in said skin potential level; convergence detection means (440) responsive to an output signal from said comparing means (430) for detecting a non-pulse like portion in said skin potential level by analyzing successive change of gradient determined by said degree from said level change detection means; storing means (480) for storing said skin potential level as a reference value when non-pulse like portion is detected by said convergence detection means; and a calculation means (490) for obtaining a set point value by multiplying said reference value by a constant smaller than one; arousal level detection means (500) for determining non-arousal state of said human body when said skin potentiol level in below said set point value and the gradient of the change of said skin potentia 1 level

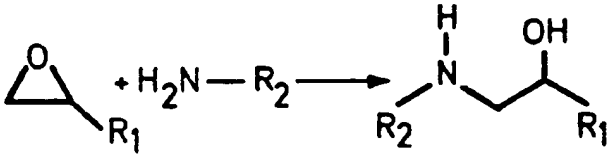
PEG-DGE

FIG.3A



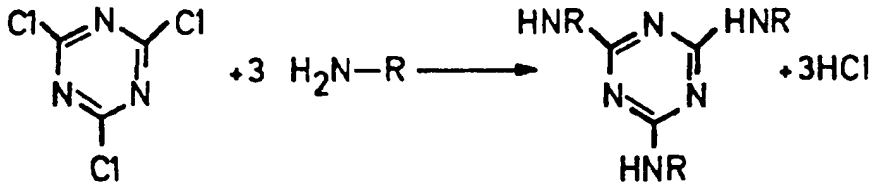
EPOXIDE REACTION WITH AN AMINE

FIG.3B



CYANURIC CHLORIDE

FIG.3C



N-HYDROXYSUCCINIMIDE ESTERS

FIG.3D

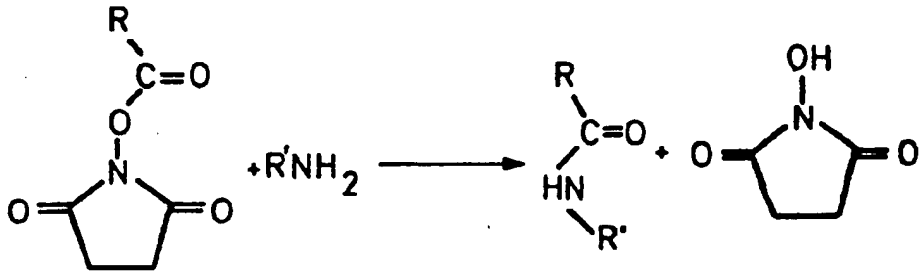
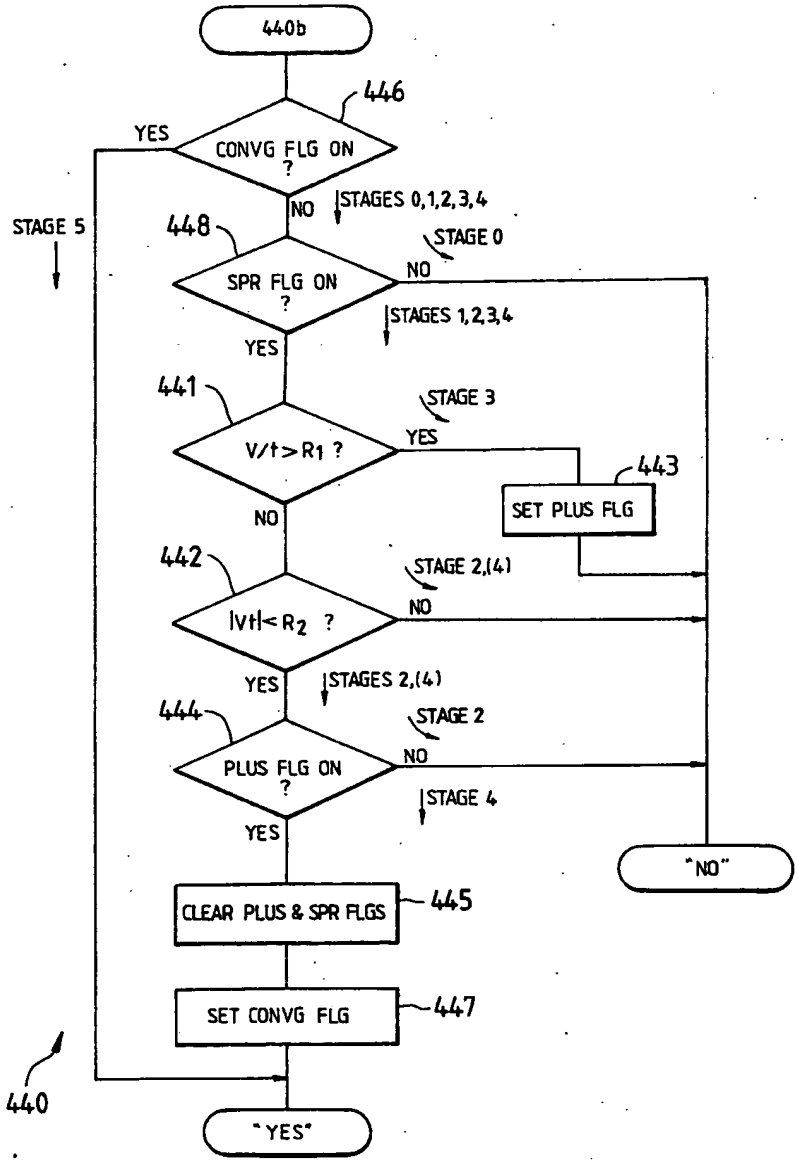
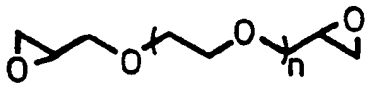


FIG. 13B


440b
446
CONVG FLG ON ?
YES
NO
STAGES 0,1,2,3,4
STAGE 5
448
SPR FLG ON ?
STAGE 0
NO
STAGES 1,2,3,4
YES
441
V/t>R1 ?
STAGE 3
YES
443
SET PLUS FLG
NO
442
|Vt|<R2 ?
STAGE 2,(4)
NO
YES
STAGES 2,(4)
444
PLUS FLG ON ?
STAGE 2
NO
STAGE 4
YES
"NO"
CLEAR PLUS & SPR FLGS
445
SET CONVG FLG
447
440
"YES"

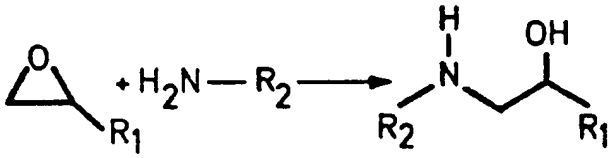
PEG-DGE

FIG.3A



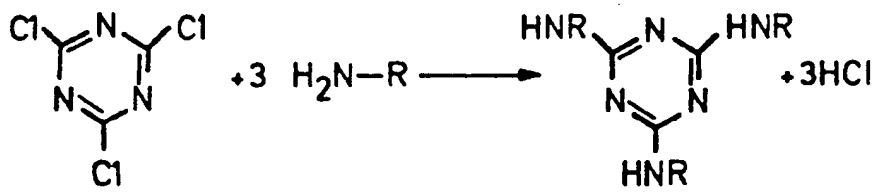
EPOXIDE REACTION WITH AN AMINE

FIG.3B



CYANURIC CHLORIDE

FIG.3C



N-HYDROXYSUCCINIMIDE ESTERS

FIG.3D

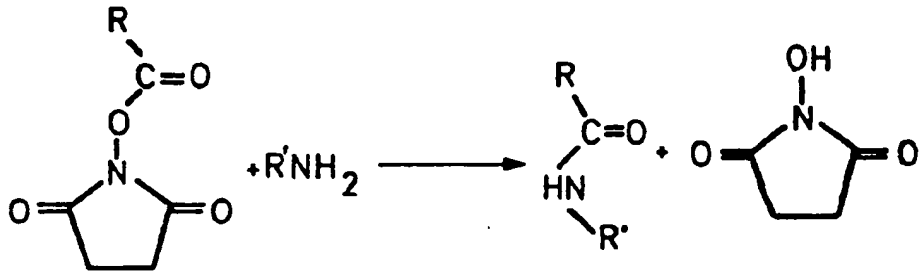


FIG. 10

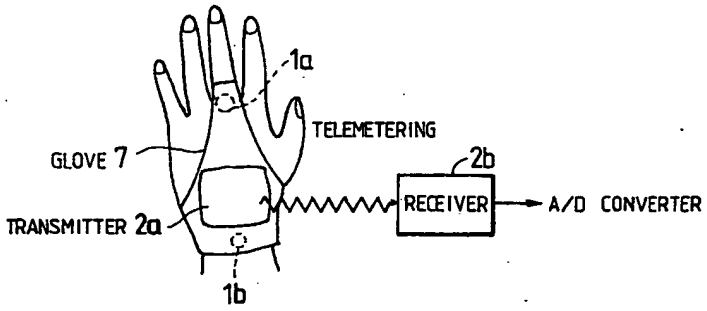


FIG. 11

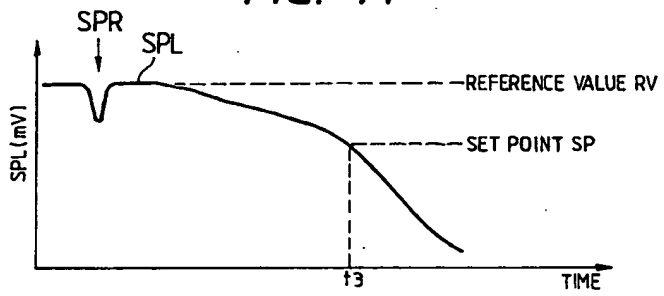
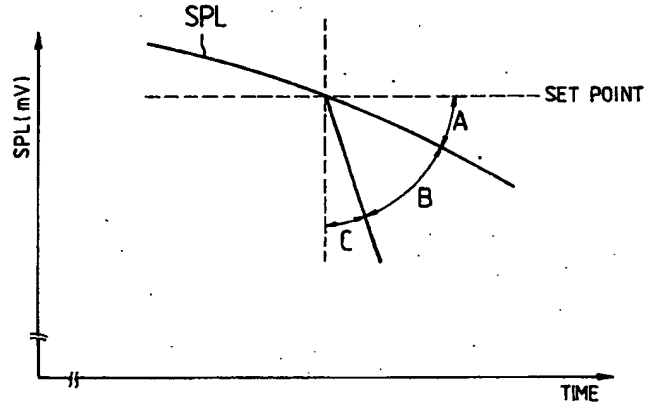
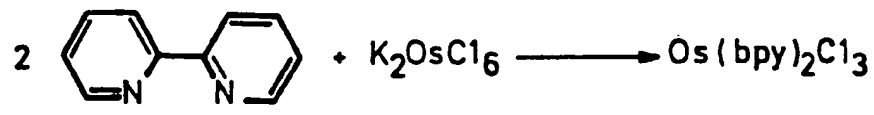
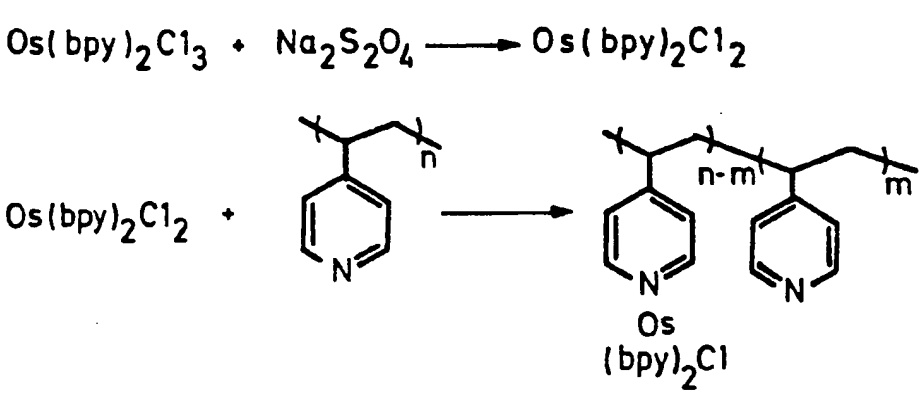


FIG. 12







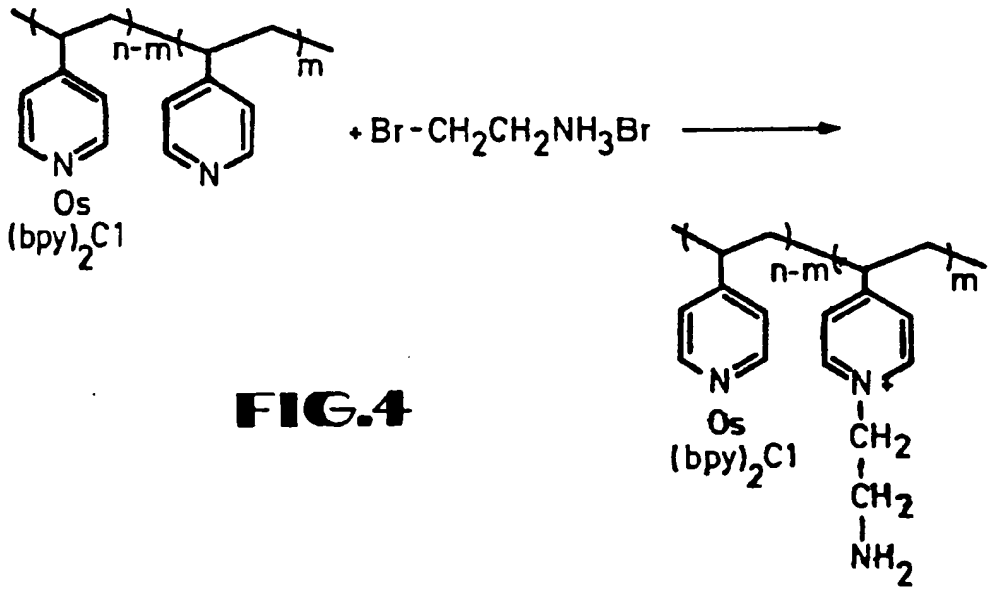


FIG.4

POLYMER C

FIG. 9A

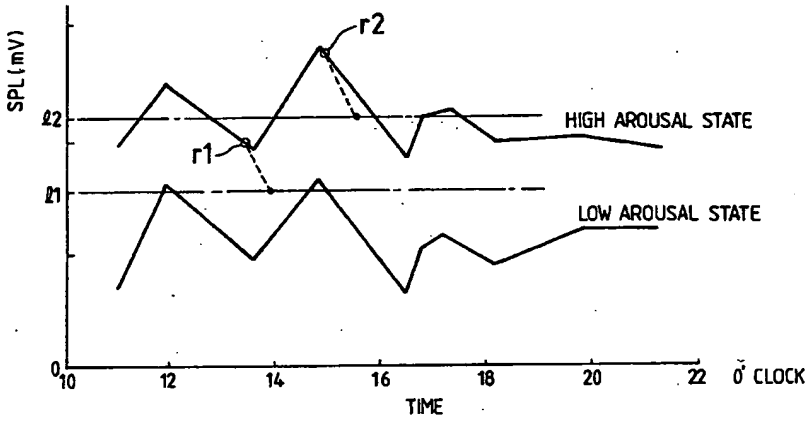

SPL (mV)
r2
r1
Ø2
Ø1
HIGH AROUSAL STATE
LOW AROUSAL STATE
0
10
12
14
16
18
20
22
0' CLOCK
TIME

FIG. 9B

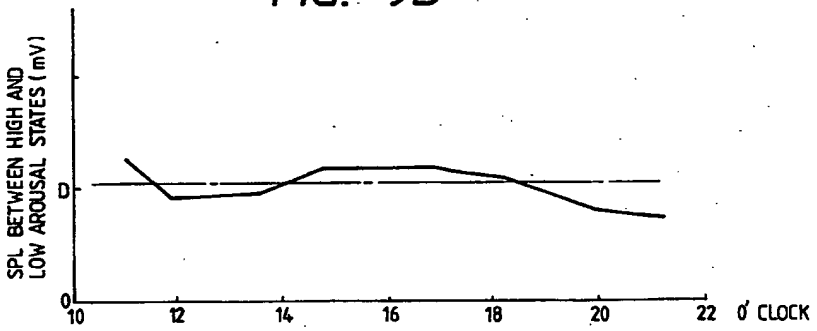

SPL BETWEEN HIGH AND LOW AROUSAL STATES (mV)
D
0
10
12
14
16
18
20
22
0' CLOCK

FIG.6

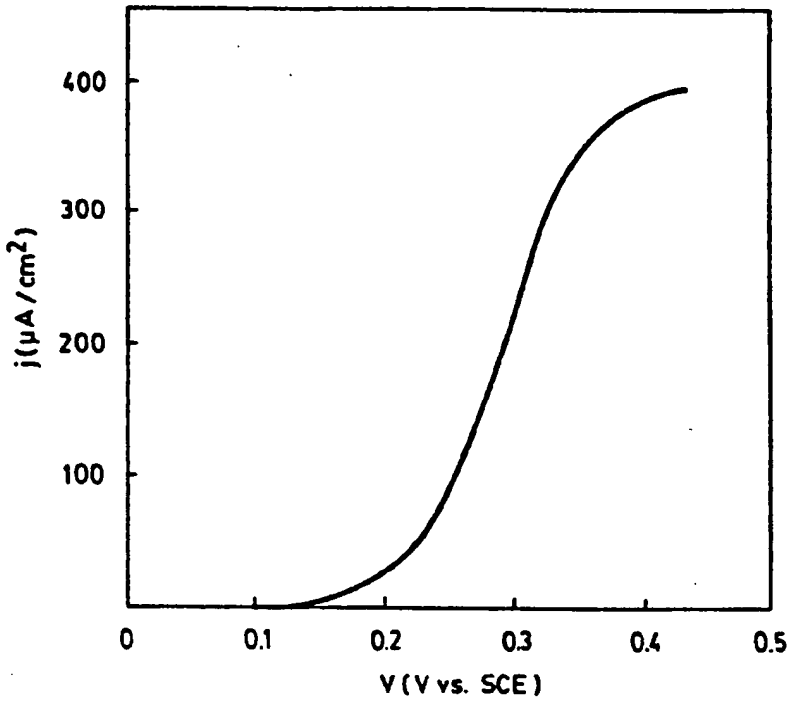
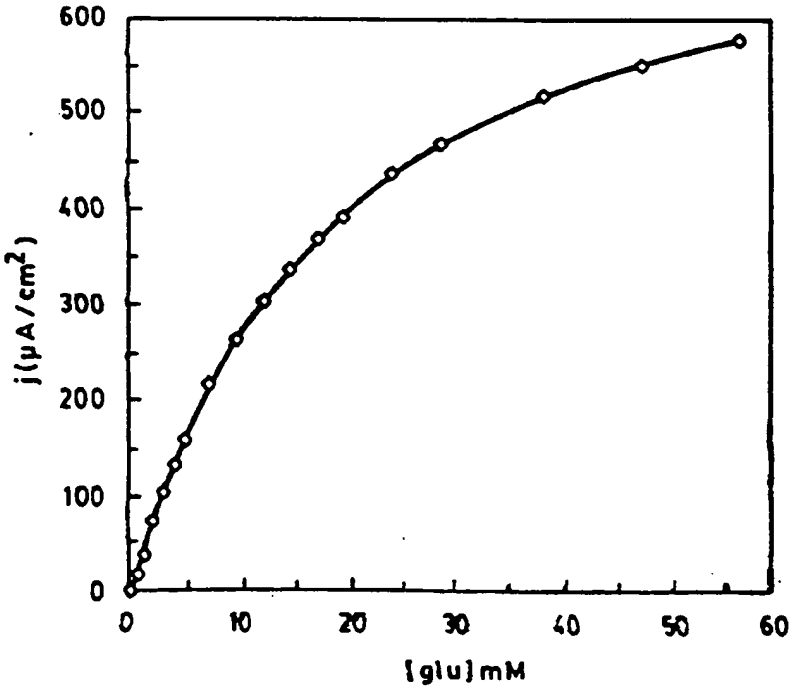

400
300
200
100
j(μA/cm²)
0
0.1
0.2
0.3
0.4
0.5
V(V vs. SCE)

FIG.7


600
500
400
300
200
100
0
j(μA/cm²)
0
10
20
30
40
50
60
[glu]mM

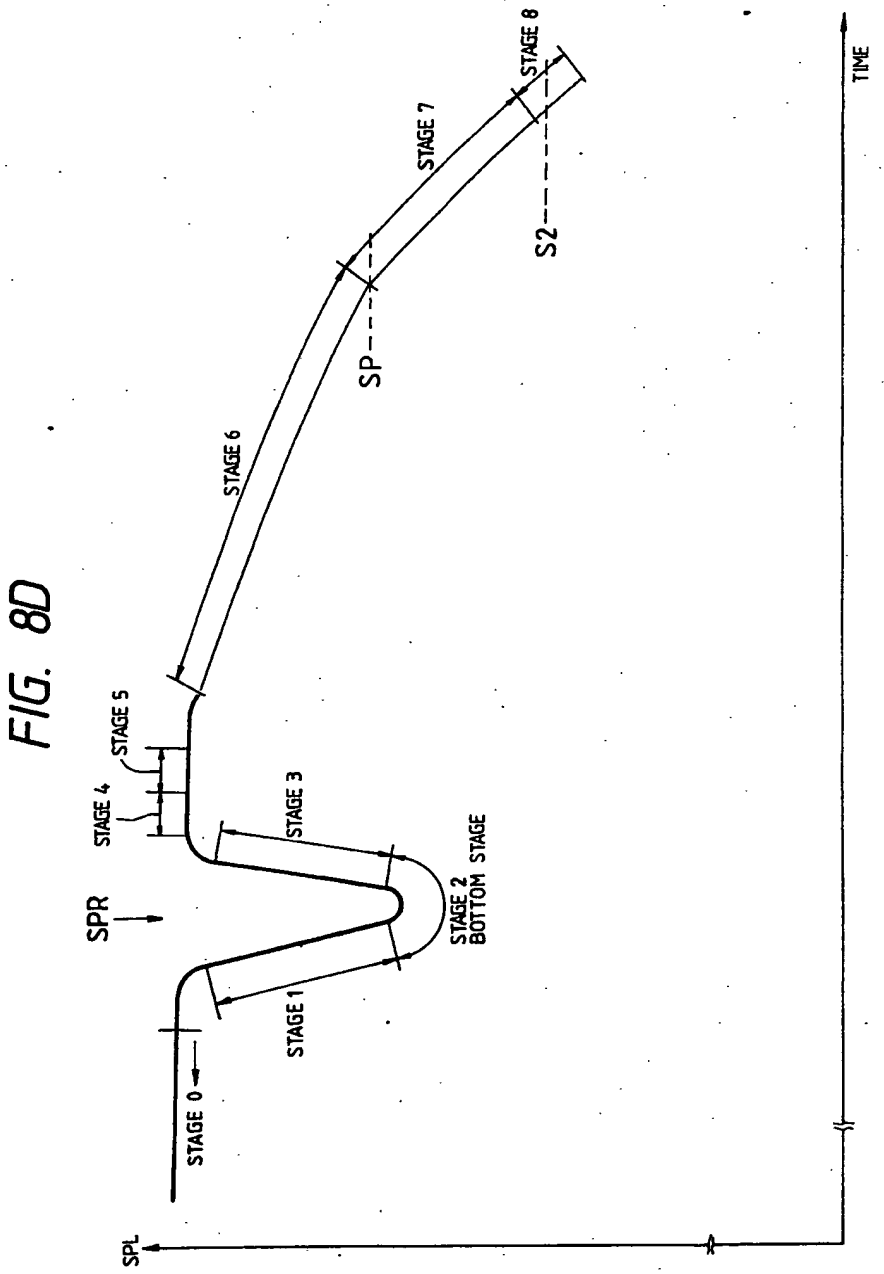

STAGE 8
STAGE 7
S2
SP
STAGE 6
STAGE 5
STAGE 4
STAGE 3
SPR
STAGE 2
BOTTOM STAGE
STAGE 1
STAGE 0
SPL
TIME

FIG. 8D

ENZYME ELECTRODES

The Government may own certain rights in this invention pursuant to Office of Naval Research Contract No. N00014-88-K-0401.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to electrodes that can selectively oxidize or reduce a biochemical in a solution. More particularly, it relates to electrodes that can translate the concentration of a biochemical to an electrical current, or can utilize an electrical current to selectively convert one biochemical to another.

2. Description of the Related Art

Enzyme based biosensors (i.e., electrochemical sensors capable of detecting the concentration of a single biochemical species in a medium containing a diverse mixture of other compounds) are used in an increasing number of clinical, environmental, agricultural and biotechnological applications. Amperometric enzyme electrodes typically require some form of electrical communication between the electrode and the active site of the redox enzyme that is reduced or oxidized by the substrate. However, the electrooxidation of a reduced site or the electroreduction of an oxidized site (the rate, of which is proportional to the concentration of the enzyme substrate) is complicated by the fact that the active site is often located deep inside an insulating protein shell. Thus, redox enzymes such as glucose oxidase do not directly exchange electrons with simple metal electrodes.

Historically, electrical communication between the enzyme and electrode has been achieved through the use of diffusing mediators. The first mediator employed for FAD-enzyme electrodes was the natural substrate of the flavin-linked oxidases, O_2. example, the reaction of glucose oxidase (GO) is

$$\text{GO-FAD} + \text{glucose} \rightarrow \text{GO-FADH}_2 + \text{gluconolactone} \quad (1)$$

$$\text{GO-FADH}_2 + O_2 \rightarrow \text{FAD} + H_2O_2 \quad (2)$$

and the first commercial amperometric glucose sensors measured either the decrease in O_2 concentration at an oxygen electrode, or the increase in H_2O_2 concentration at a platinum electrode.

There were several problems associated with such devices: (1) the H_2O_2 degraded the enzyme. Nature alleviates this problem through the use of a second enzyme, usually catalase, which is present in high concentrations in cells and catalyses the disproportionation of the H_2O_2; (2) the electrode current depended on the concentration of both the enzyme substrates, i.e., both glucose and O_2; (3) measurement of the H_2O_2 concentration required both a highly catalytic electrode (e.g., Pt) and a potential (ca. 0.7 V vs. SCE) substantially positive of the reversible potential for the FAD/-$FADH_2$ couple (E° is approximately equal to -0.4 V vs. SCE). This resulted in large spurious currents due to a number of easily oxidized species in the system to be measured. Because of (2) and (3), the amperometric biosensors were not adequately substance-specific.

The most recent devices have employed small diffusing redox shuttles (Ox/Red) such as ferrocenes, quinones, ruthenium ammines, components of organic metals, and octacyanotungstates. In such electrodes, reaction (1) above is followed by

$$\text{GO-FADH}_2 + \text{Ox} \rightarrow \text{GO-FAD} + 2\,\text{Red} + 2H^+ \quad (3)$$

where the reduced form of the shuttle (Red) is subsequently electrooxidized. Catalase can be added to the system to protect the enzyme from H_2O_2. The potential at which these electrodes operate is only slightly positive of the formal potential of the shuttle, and a highly active noble metal electrode is no longer required for the reaction. Thus, the spurious currents due to competing species may be reduced. Still, in an oxygen containing medium, there is a competition between the oxidized form of the shuttle (Ox) and oxygen for the reduced form of the enzyme ($GO\text{-}FADH_2$), equations (2) and (3). Thus, the electrode current will be independent of the oxygen concentration only insofar as the shuttle can compete effectively with O_2.

Enzyme electrodes such as those just described generally require that the enzyme and shuttle be confined to the proximity of the electrode surface. The small shuttles commonly employed can, however, readily diffuse through the membranes that are needed to contain the enzyme, but permit the passage of the enzyme's substrate, e.g., glucose. Recently, a polymeric redox "wire" based on the poly(vinyl-pyridine) (PVP) complex of $Os(bpy)_2Cl$ (abbreviated POs^{30}; the bpy of the complex is 2,2'-bipyridine) has been introduced which electrically connects the enzyme to the electrode yet, by virtue of its molecular size, remains confined behind the enzyme-containing membrane. This polycationic redox polymer forms electrostatic complexes with the polyanionic glucose oxidase in a manner mimicking the natural attraction of some redox proteins for enzymes, e.g., cytochrome c for cytochrome c oxidase.

Enzyme electrodes now in use are of several different types. One type of electrode amperometrically measures the oxygen content of gas streams entering and leaving a reactor containing the substrate and its enzyme. If oxygen is involved in the substrate's enzymatic oxidation, its level is depleted and the substrate concentration can be deduced from the decrease in the oxygen content of the gas.

With a second type of enzyme electrode, a natural electroreactive product of the enzyme-catalyzed reaction is amperometrically monitored. For example, the enzymatic reaction of substrates like glucose or lactate with oxygen, catalyzed by some oxidases, produces hydrogen peroxide. Hydrogen peroxide can be electrooxidized and thereby the substrate concentration over a certain range can be translated into a current.

In a third type of enzyme electrode, a non-natural redox couple mediates electron transfer from the substrate-reduced enzyme to the electrode. In this scheme, the enzyme is reduced by its natural substrate at a given rate; the reduced enzyme is in turn, rapidly oxidized by a non-natural oxidizing component of a redox couple that diffuses into the enzyme, is reduced, diffuses out and eventually diffuses to an electrode where it is oxidized. Here again, the oxidation current can be related to the concentration of the substrate. A specific example of such a redox mediator is the ferricinium carboxylate/ferrocene carboxylate couple that diffusionally mediates electron transfer from glucose reduced glucose oxidase to a carbon electrode.

Most natural enzymes are not directly oxidized at electrodes, even if the latter are maintained at strongly

FIG. 8B

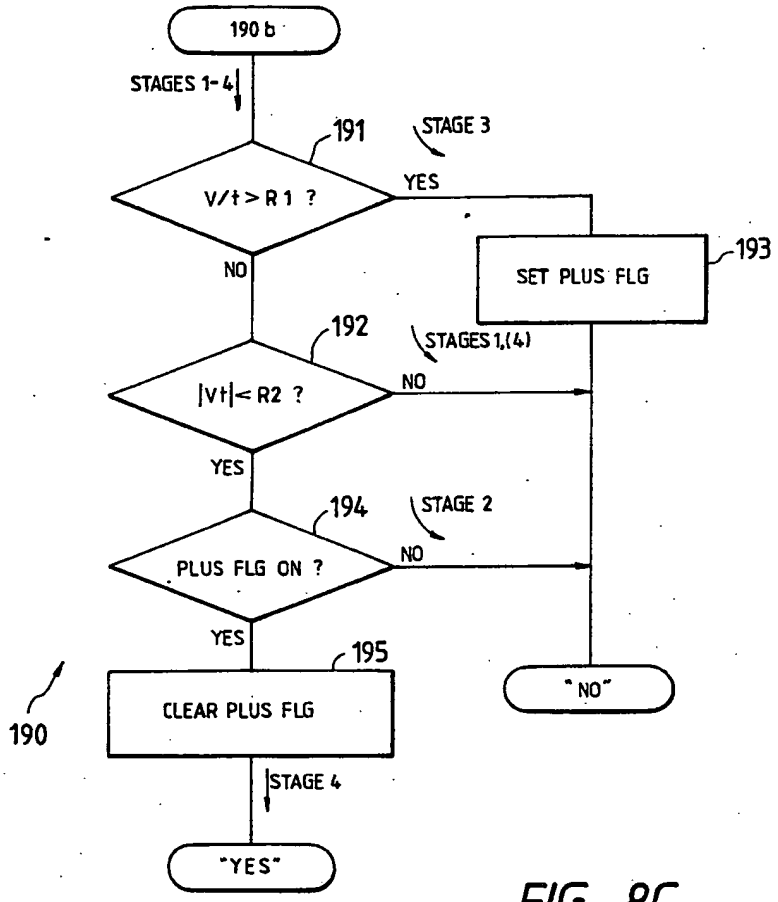
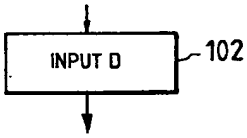

190 b
STAGES 1-4
191
STAGE 3
V/t > R 1 ?
YES
NO
193
SET PLUS FLG
192
STAGES 1,(4)
|Vt| < R2 ?
NO
YES
194
STAGE 2
PLUS FLG ON ?
NO
YES
195
CLEAR PLUS FLG
"NO"
190
STAGE 4
"YES"

FIG. 8C


INPUT D
102

oxidizing potentials, without being destroyed. Also they are not reduced at strongly reducing potentials without being decomposed. It has, however, been shown that enzymes can be chemically modified by binding to their proteins redox couples, whereupon, if in the reduced state, they transfer electrons to an electrode. Thus, amperometric glucose sensors have been made with glucose oxidase to which ferricinium/ferrocene functions have been chemically bound. It has also been shown that when redox polycations in solution electrostatically complex polyanionic enzymes, electrons will flow in these complexes from the substrate to the enzyme, and from the enzyme through the redox polymer, to an electrode. Glucose electrodes have also been built with these complexes.

The current produced at a given substrate level can depend on the concentration of the active enzyme molecules. It has been shown that natural reaction products, like hydrogen peroxide, deactivate the enzyme. Enzymes are also continuously denatured. It has been shown that the denaturing of enzymes can be retarded by embedding the enzyme in a rigid three-dimensional polymer structure. It has been suggested that such embedding fixes the protein structure of the enzyme, preventing conformational changes that result in its eventual denaturing. For example, chymotrypsin has been stabilized by embedding it in crosslinked poly(methyl methacrylate).

SUMMARY OF THE INVENTION

Broadly, the invention relates to materials (and films formed from such materials) which include at least two components that can combine to form a three dimensional moleoular structure. At least one of the components comprises a redox compound, and at least one other component comprises an oxidoreductase (hereinafter referred to as a redox enzyme). The resulting three dimensional molecular structure has multiple redox centers and has the redox enzyme bound within.

When such materials are coated onto a surface, the three dimensional molecular structure provides electrical contact between that surface and the redox enzyme. In the three dimensional structure sigma bonds dominate the polymer's backbone, wherefore electron delocalization is limited.

The term "three dimensional molecular structure" as used herein means a structure in which covalent chemical bonds extend in three dimensions. The term is not meant to include a three dimensional structure formed by mere physical bonding of molecules, for example through Van der Waals forces.

The term "redox compound" is used herein to mean a compound that can be oxidized and reduced. The redox compound may have one or more functions that are reducible and oxidizable. Stated another way, the term "redox compound" means a compound which contains one or more redox centers, "redox center" meaning a chemical function that accepts and transfers electrons.

In one embodiment, a material is provided comprising a redox enzyme, a crosslinking agent, and a crosslinkable compound capable of reacting with the crosslinking agent and the redox enzyme. Either the crosslinkable compound or the crosslinking agent, or both, have one or more redox centers. In an alternative embodiment, a material is provided comprising a redox enzyme and a redox compound having two or more functional groups capable of reacting with the enzyme (i.e. a redox compound capable of crosslinking with the enzyme).

When the compounds of each embodiment are mixed together under appropriate conditions, a chemical reaction takes place resulting in the formation of a crosslinked (three-dixensional) redox polymer, with the redox enzyme bound within the crosslinked redox polymer network.

It should be noted that in the alternative embodiment discussed above, the redox enzyme itself is used as the crosslinking agent to crosslink the redox compound into a three dimensional molecular structure. Most (if not all) enzymes have multiple (more than two) functions that can react. Examples of such enzyme functions are amine, phenol, tryptophane, thiol, and imidazole functions.

By "bound within" it is meant that the redox enzyme is contained or incorporated within the crosslinked polymer structure in such a manner that the enzyme will not tend to diffuse out of the structure. Thus, for example, the enzyme may be chemically (covalently) bonded, electrostatically bonded, or hydrogen bonded to the polymer, and not simply physically bound or trapped within cavities of the polymer surface.

The term "crosslinkable compound" is used herein to mean a compound containing at least two groups (i.e., a bi-or-multifunctional compound) capable of reacting with itself or another bi-or-multifunctional compound, resulting in a macromolecule. The term "crosslinking agent" is used herein to mean a compound containing at least two functional groups capable of reacting with and crosslinking other compounds, i.e. it is the substance that crosslinks the crosslinkable compound.

One particularly important application of these materials is in the area of amperometric biosensors. However, it should be understood that these materials have other applications where it is desired to electrically connect redox enzymes to electrodes, as in the electrosyrthesis of biochemicals.

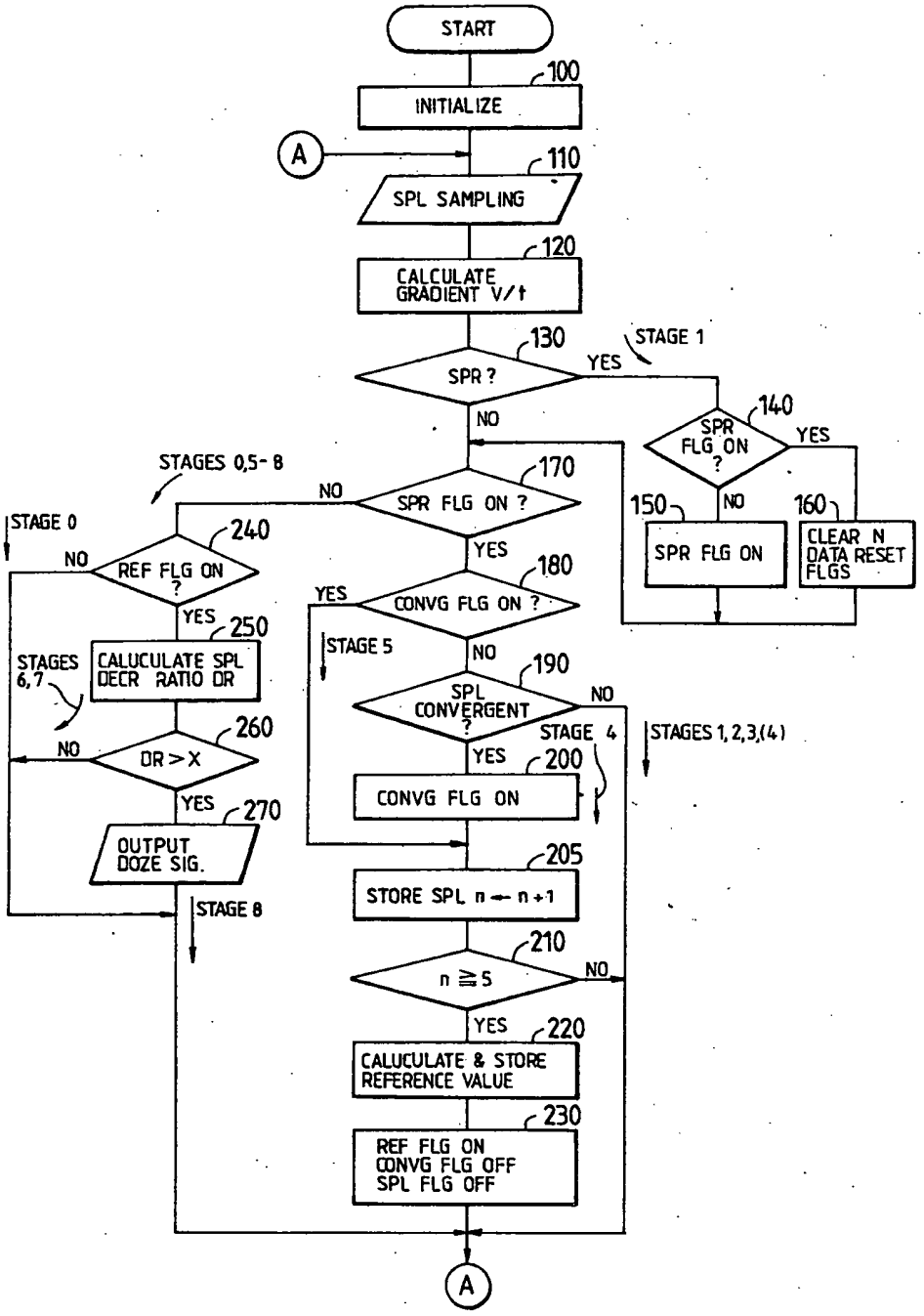
In another broad aspect of the invention, an electrode is provided having a surface coated with a film of a material of the class described above. The term "film" is used broadly to include any coating or layer of the material regardless of thickness or method of application.

In another broad aspect, the present invention provides for the construction of enzyme electrodes employing this class of materials. This process may involve the mixture of the enzyme and the various polymer components in a common solution followed by the application of the solution to an electrode surface. Various application methods may be used, including (1) addition of drops of the solution onto the electrode surface; (2) dipcoating; (3) spincoating, or (4) spraying the solution onto the electrode surface. The application step is followed by a curing step such as drying in air or vacuum.

Alternatively, the process may involve the addition of the enzyme and polymer components in separate solutions to the surface of the electrode, mixing, and then curing in air or vacuum.

The preferred crosslinkable compounds for use in this invention are hydrophilic, containing chemical groups such as alcohols, carboxylic acids, amines, amides, sulfonates, sulfates, phosphates and phosphonates. Such groups tend to promote the solubility of the components in water which facilitates contact with the water soluble enzymes. Such groups may also improve the

FIG. 8A


START
100
INITIALIZE
A
110
SPL SAMPLING
120
CALCULATE GRADIENT V/t
130
SPR ?
YES
STAGE 1
NO
140
SPR FLG ON ?
YES
NO
150
SPR FLG ON
160
CLEAR N DATA RESET FLGS
STAGES 0,5-8
NO
170
SPR FLG ON ?
YES
STAGE 0
240
REF FLG ON ?
NO
YES
250
CALUCULATE SPL DECR RATIO DR
STAGES 6,7
260
DR > X
NO
YES
270
OUTPUT DOZE SIG.
STAGE 8
180
CONVG FLG ON ?
YES
NO
STAGE 5
190
SPL CONVERGENT ?
NO
YES
STAGE 4
STAGES 1,2,3,(4)
200
CONVG FLG ON
205
STORE SPL n ← n+1
210
n ≧ 5
NO
YES
220
CALUCULATE & STORE REFERENCE VALUE
230
REF FLG ON
CONVG FLG OFF
SPL FLG OFF
A

stability of the immobilized enzyme against denaturation.

The redox compounds (or redox centers contained within compounds) used in this invention may be either organic or inorganic. Transition metal complexes with organic ligands such as bipyridine or cyclopentadiene are often preferred as redox centers because of their chemical stability in various oxidation states and their facile electron transfer kinetics. Typical examples of such complexes are the polypyridine complexes of di-or trivalent osmium ions and the various derivatives of ferrocene (bis-cyclopentadienyl iron) or cobaltocene (bis-cyclopentadienyl cobalt). However, a number of organic redox centers may also be employed. The various derivatives of viologen (N,N'-bis alkyl-4,4'-bipyridine) constitute typical examples of this class.

The preferred crosslinking agents are water soluble compounds that react under conditions where most enzymes are stable, that is around pH 7 and room temperature. Included in this category of crosslinking agents are multifunctional epoxides, aldehydes, imidoesters, N-hydroxysuccinimide esters and carbodiimides. A number of reagents with limited solubility in water may also be used by dissolving them in a water-miscible organic solvent such as acetone, methanol, acetonitrile or dimethylformamide. Included in this category are reagents such as cyanuric chloride, tetrachlorobenzoquinone, benzoquinone and tetracyanoquinodimethane. These reagents may react with one or more types of functions including amines, alcohols, thiols and carboxylic acids which may be present on the surface of enzymes and may also be included in the structure of the redox compound.

The electrodes to which the crosslinked redox polymer is applied can be made of any of a number of metals, semi-metals, or semiconductors. For example, gold, platinum, glassy carbon, or graphite electrodes may be used.

In one preferred embodiment, osmium bis(2,2'bipyridine) dichloride is coordinated to a poly(vinyl-pyridine) chain forming approximately one osmium bis(bipyridine) vinylpyridine chloride complex per five vinylpyridine units. The remaining vinylpyridines are quaternized with bromoethylamine hydrobromide, leading to a very hydrophilic redox polymer containing pendant ethylamine groups. This polymer may be dissolved in an aqueous solution containing the enzyme and a water soluble diepoxide, such as poly(ethylene glycol diglycidyl ether). Upon applying the solution onto an electrode surface and drying in air or vacuum, the epoxide may react with both the ethylamine pendant groups of the redox polymer and the surface lysine residues of the enzyme. This results in an enzyme-containing crosslinked redox polymer film on the electrode surface.

The method of operation of such an enzyme electrode may be illustrated using a glucose electrode as an example. Upon immersion of the electrode into a solution containing glucose, the glucose diffuses into the film where it may react with the glucose oxidase enzyme forming gluconolactone and the reduced form of the enzyme. The reduced enzyme may then be oxidized by the osmium complex-containing polymer. Electrons are subsequently transferred through the polymer to the electrode. Thus, an electrical current proportional to the concentration of the enzyme substrate is achieved.

Electrons from a substrate-reduced enzyme can be transferred either to the enzyme's natural re-oxidizer (oxygen in the case of glucose oxidase, lactate oxidase and other flavoenzymes) or, via the redox-centers of the polymer to the electrode. Only the latter process contributes to the current. Thus, it is desirable to make the latter process fast relative to the first. This can be accomplished by (a) increasing the concentration of the redox centers (e.g. the number of osmium complexes) in the film, or (b) assuring that these centers are fast, i.e. that they are rapidly oxidized and reduced. It is also desirable to make the redox centers oxidizing with respect to the reduced enzyme. This often increases the rate of transfer of electrons to the electrode.

However, it is also true that the higher the oxidation potential of the redox couple, the more extraneous compounds may be oxidized by it, that is, the less selective is the electrode. Thus, there is an optimum range of oxidation potential for the redox couple for any given application. Similar arguments hold for electrodes which will be used in the reduction of enzymes.

It should be appreciated that this description applies equally to the operation of a biosensor (in the above case, a glucose sensor) or an electrosynthesizer of biochemicals (in this case, gluconolactone, the product that is electrosynthesized). Thus, although in practice, the two devices may be differently configured, the scope of the present invention encompasses both biosensors and bioelectrosynthesizers, and related devices.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic drawing of a crosslinked redox polymer-enzyme electrode as provided by the present invention.

FIG. 2 shows several examples of redox centers bound to multifunctional compounds capable of forming crosslinked polymers when reacted with crosslinking agents, including enzymes or other multifunctional compounds, in accordance with the present invention.

FIG. 2A shows a crosslinkable redox compound, Polymer A.

FIG. 2B shows a corsslinkable redox compound, Polymer B.

FIG. 2C shows a crosslinkable redox compound, Polymer C.

FIG. 2D shows a crosslinkable redox compound, Polymer D.

FIG. 2E shows a crosslinkable redox compound, Polymer E.

FIG. 2F shows a crosslinkable redox compound, Polymer F.

FIG. 2G shows a crosslinkable redox compound, Polymer G.

FIG. 3 shows several examples of crosslinking agents used by the present invention and some of the typical reactions which they undergo.

FIG. 3A shows the epoxide crosslinking agent PEG-DGE.

FIG. 3B shows reaction of PEG-DGE with amine.

FIG. 3C shows reaction of the crosslinking agent cyanuric chloride with amine.

FIG. 3D shows reaction of the crosslinking agent N-Hydroxysuccinimide with an amine.

FIG. 4 shows a synthetic scheme for one of the preferred crosslinkable redox polymers as provided by the present invention.

FIG. 5 shows a number of cyclic voltammograms of a crosslinked redox polymer film containing glucose oxidase prepared according to the present invention. There is no glucose in solution. Scan rates (mV/s)(a) 10, (b) 20, (c) 50, (d) 100, (e) 200.

FIG. 6

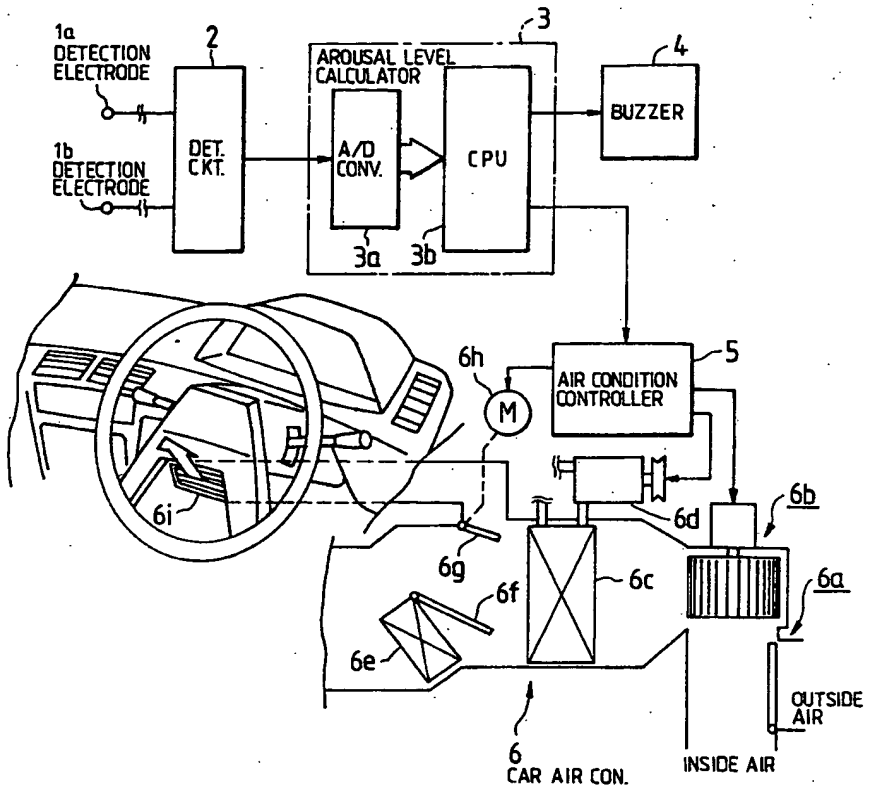

1a
DETECTION
ELECTRODE
1b
DETECTION
ELECTRODE
2
DET.
CKT.
3
AROUSAL LEVEL
CALCULATOR
A/D
CONV.
CPU
3a
3b
4
BUZZER
5
AIR CONDITION
CONTROLLER
6h
M
6i
6g
6f
6e
6c
6d
6b
6a
OUTSIDE
AIR
INSIDE AIR
6
CAR AIR CON.

FIG. 7

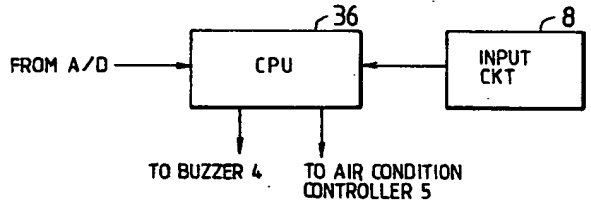

36
8
FROM A/D
CPU
INPUT
CKT
TO BUZZER 4
TO AIR CONDITION
CONTROLLER 5

FIG. 6 shows a cyclic voltammogram of the film used in FIG. 5 after addition of 40 mM glucose. Scan rate 5 mV/s.

FIG. 7 shows a typical response curve (current density versus substrate concentration) for a glucose electrode prepared in accordance with the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The materials and processes provided by the present invention, the crosslinked redox polymers and the incorporation of redox enzymes in them, have particularly important applications in the manufacture of enzyme electrodes of the type illustrated in FIG. 1. These electrodes may be used in such applications as amperometric biosensors and the electrosynthesis of biochemicals.

There are several advantages to an enzyme electrode system based on a crosslinked redox polymer. First, the use of crosslinked films on the electrode surface eliminates the requirement for a membrane which is often required in conventional systems to confine the enzyme to a small volume close to the electrode surface. Thus, the use of crosslinked redox films tends to simplify the design and the manufacture of the enzyme electrode. Second, the process by which the electrodes are produced is relatively simple, reproducible and can be easily automated. Third, the enzyme may be stabilized by its interaction with the polymer matrix, thus retarding thermal denaturation. Also, it may be physically protected from attack by proteases in solution which are too large to diffuse through the polymer film. Fourth, the versatility of these materials allows the tailoring of properties for specific applications. For example, the redox potential, the hydrophilicity and the charge on the polymer may be adjusted as may the crosslinking method. Fifth, the transport of interfering electroreactive substances to the electrode surfaces and/or their adsorption on these surfaces can be retarded by appropriate design of the polymer. Sixth, the resulting electrodes are in general mechanically rugged and typically exhibit excellent stability during storage. Seventh, although enzymes are known to rapidly denature on many surfaces, the polymer apparently tends to protect the enzymes from the surface of the electrode. Thus, virtually any electrode surface may be used for these enzyme electrodes. Additionally, such polymers in general appear to be substantially biocompatible.

In one preferred embodiment, the water soluble crosslinking agent polyethylene glycol diglycidylether (PEG-DGE, FIG. 3) is used to react with redox compounds with amine functions and with amine functions of the lysine groups of the enzyme. The reaction between epoxides and amines is particularly advantageous since the reaction (1) releases no low molecular weight species; (2) does not greatly change the local pH; (3) does not greatly change the charge on either the redox compound or the enzyme; and (4) is compatible with a number of different enzymes. PEG-DGE is also commercially available in a number of chain lengths. The reaction between PEG-DGE and amines proceeds very slowly in dilute aqueous solution. Thus, all the reactants may be combined in a single solution before the application step which greatly simplifies the manufacture of the electrodes. The crosslinking reaction may then proceed to completion when the solution is dried on the surface of the electrode. The cure time for the film is 24 to 48 hours at room temperature.

An enzyme electrode as provided by the present invention is shown schematically in FIG. 1. The electrode 10 has a surface 12 which is coated with a crosslinked redox polymer film 14. A redox enzyme 16 is bound to the polymer 14. The polymer 14 electrically connects the electrode 10 to the enzyme 16.

Various preferred crosslinkable compounds containing redox active centers are shown in FIG. 2. Polymer A and Polymer F are representative of that class of compounds which require only the addition of enzymes to form crosslinked films, i.e. the enzyme is the only required crosslinking agent. The other compounds are representative of that class of compounds which do not react directly with chemical functions on the enzyme. They therefore require a separate crosslinking agent such as those illustrated in FIG. 3.

FIG. 3 shows three representative classes of crosslinking agents, and their reactions with a typical organic compound having an amine group, represented as RNH_2. The crosslinking agents shown are an epoxide (e.g. PEG-DGE), cyanuric chloride, and an N-Hydroxysuccinimide.

Characteristic cyclic voltammograms of a film containing Polymer F, glucose oxidase and triethylenetetraamine in the absence of glucose on glassy carbon are shown in FIG. 5. The almost symmetrical shape of the oxidation and reduction waves, and the fact that the peak currents do not decrease over time show that the polymer film is strongly attached to the electrode surface and in good electrical contact with it. The fact that the peak shape changes very little upon increasing the scan rate from 10 mV/s to 200 mV/s is evidence for fast electron transfer through the polymer film.

FIG. 6 shows a cyclic voltammogram of the same film as FIG. 5 after the addition of glucose to a final concentration of 40 mM. A catalytic oxidation is exhibited as the electrons are transferred from the glucose-reduced enzyme to the redox polymer and from the redox polymer to the electrode.

A typical response curve of a Polymer C-glucose oxidase-PEG-DGE film is shown in FIG. 7. As the glucose concentration is increased the current response follows the characteristic Michaelis-Menten behavior of the enzyme.

EXAMPLES

The following examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all features and all embodiments of the present invention, and should not be construed as limiting the claims presented herein.

EXAMPLE 1

The synthetic scheme for this example is illustrated FIG. 4. cis - Bis(2,2'-bipyridine-N,N') dichloroosmium (II) ($Osbpy_2Cl_2$) was prepared by a standard literature procedure (Lay, P.A.; Sargeson, A.M.; Taube, H., Inorg. Syn. 1986, 24, 291). Polyvinylpyridine (PVP), nominal molecular weight 50,000, was purchased from Polysciences, Inc. and purified three times by dissolution in methanol and precipitation with ether. 0.494 gram $Os(bpy)_2Cl_2$ and 0.430 gram PVP were added to 18 mls. of ethylene glycol in a round bottom flask under nitrogen. The mixture was slowly heated to reflux (196° C.) and maintained at reflux for about 105 minutes. It was then cooled to room tempera-

FIG. 4

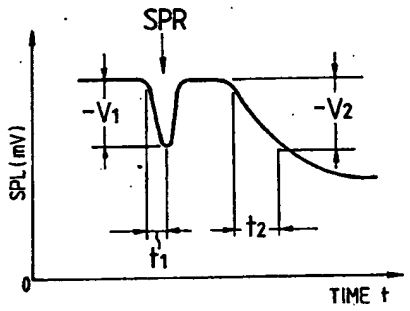
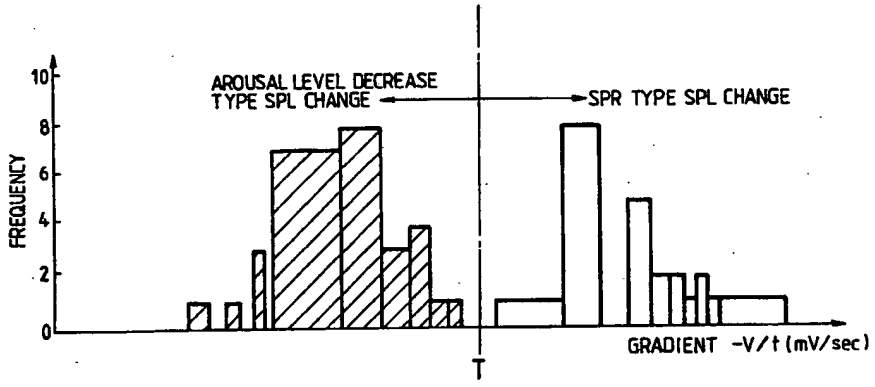

SPR
-V1
-V2
SPL (mV)
t1
t2
0
TIME t

FIG. 5


AROUSAL LEVEL DECREASE TYPE SPL CHANGE
SPR TYPE SPL CHANGE
FREQUENCY
10
8
6
4
2
0
GRADIENT -V/t (mV/sec)
T

ture and 30 mls. of dimethylformamide (DMF) was added. 1.5 gram bromoethylamine hydrobromide was added to the mixture which was then stirred at about 35° C. overnight. The polymer solution was then poured into a rapidly stirred solution of acetone and the precipitate was filtered, washed with acetone and stored in a vacuum dessicator. The approximate structure of this polymer is shown in FIG. 2 (Polymer C).

Three solutions were made up in aqueous 10 mM HEPES buffer at pH 8:1:

Solution 1 contained 10 mg/ml polymer C

Solution 2 contained 5 mg/ml glucose oxidase

Solution 3 contained 2.7 mg/ml PEG-DGE

The enzyme containing solution was made up fresh every day; the other two solutions were stable for at least one month. 15 microliters of solution 1, 15 microliters of solution 2 and 5 microliters of solution 3 were thoroughly mixed in a vial and 3 microliters of the mixture was deposited onto a glassy carbon disk electrode (4.5 mm in diameter). The electrode was then placed in a vacuum dessicator for 24 hours. Upon exposure to solutions containing high concentrations of glucose (≧60 mM), such electrodes commonly exhibited current densities of 400–1100 microA/cm^2 at a potential in the 0.35–0.45 volt range measured relative to the potential of the Standard Calomel Electrode (SCE). In the absence of glucose, the current density was approximately 1 microA/cm^2.

EXAMPLE 2

The procedure of Example 1 was repeated but cyanuric chloride was used as the crosslinking agent in place of PEG-DGE. In this case the polymer and enzyme were made up in 100 mM phosphate buffer solution at pH 7.1. 2 microliters each of the polymer and enzyme solution were mixed on the electrode surface with 0.5 microliters of an acetonitrile solution of cyanuric chloride (20 mM). This crosslinking reaction is quite fast and the electrode films required a curing time of only about 30 minutes in air or vacuum. Upon exposure to solutions containing high concentrations of glucose (≧60 mM), such electrodes commonly exhibited current densities of 80–120 microA/cm^2 at a potential in the 0.35–0.45 volt range measured relative to the SCE. In the absence of glucose, the current density was approximately 1 microA/cm^2.

EXAMPLE 3

9.6 mls. bromoacetyl chloride was dissolved in 120 ml of methylene chloride and cooled to 0° C. under nitrogen. 13.4 gram N-hydroxysuccinimide and 11.8 gram triethylamine were dissolved in 50 ml of methylene chloride and slowly dripped into the cold solution of acid chloride over 30 minutes. The solution was stirred for an additional 20 minutes. Then ice water was added, the phases were separated, the organic phase was washed two more times with ice water, once with saturated sodium chloride solution and dried over magnesium sulfate. The solution was concentrated under vacuum until crystals started to appear. Then hexane was added and the solution was cooled to 0° C. The crystals of bromoacetoxysuccinimide were filtered and dried in a vacuum dessicator.

0.507 gram Osbpy$_2$Cl$_2$ and 0.507 gram PVP were reacted in refluxing ethylene glycol for 30 minutes, the solution was then cooled, 20 mls. of acetone was added and the mixture was poured into rapidly stirred ethyl acetate. The resulting polymer (PVP-Osbpy$_2$Cl) was filtered and dried in vacuum.

0.31 gram PVP-Osbpy$_2$Cl and 0.12 gram 2-bromoethanol were dissolved in 25 mls. DMF and refluxed for 30 minutes. Then about 1 gram (a large excess) of bromoacetoxysuccinimide was added and the solution was heated at 40° C. for about 2 hours. It was then cooled, poured into stirred acetone, filtered and stored in a vacuum dessicator. This procedure led to a polymer whose approximate structure is shown in FIG. 2 (Polymer A).

A solution of 22 mg/ml Polymer A in deionized water was prepared immediately before use. Another solution in 0.1 M HEPES buffer was prepared containing 22 mg/ml glucose oxidase and 1.1 microliter/ml catalase solution. 10 microliters of each solution were mixed on the surface of a 6 mm diameter graphite rod electrode and cured at room temperature for 24 hours in vacuum. In a solution containing 31 mM glucose, this electrode exhibited a current density of about 300 microA/cm^2 when held at a potential of 0.45 volt relative to the SCE. Under these conditions, but in the absence of glucose, the electrode gave a background current density of about 4 microA/cm^2. In such films the polymer probably reacts with the lysines on the enzyme surface resulting in a crosslinked film. Small amounts of an additional polyamine, for example, triethylenetetraamine, may also be added to such films to improve their physical properties.

EXAMPLE 4

The synthetic procedure of Example 3 was repeated with the substitution of 3-bromopropionyl chloride for bromoacetyl chloride. The resulting polymer containing esters of hydroxysuccinimide was dispersed in DMF and a large excess of ethanolamine was added. The mixture was stirred overnight at room temperature, filtered and poured into stirred tetrahydrofuran (THF). The precipitate was filtered and dried. This procedure led to a polymer whose approximate structure is shown in FIG. 2 (Polymer B).

Three solutions were made up in 10 mM HEPES at pH 8.4:

Solution 1 contained 10 mg/ml Polymer B

Solution 2 contained 8 mg/ml glycerol-3-phosphate oxidase

Solution 3 contained 4 mg/ml cyanuric chloride in acetonitrile

5 microliters each of solutions 1 and 2 were mixed on the surface of a glassy carbon disk electrode with 2 microliters of solution 3. The electrode was dried in vacuum for 50 minutes. In the presence of 10 mM L-alpha-glycerophosphate this electrode exhibited a current density of 30 microA/cm^2 when held at a potential of 0.45 volts relative to the SCE reference. In the absence of a L-alpha-glycerophosphate, the current density was 1.1 microA/cm^2 at the same potential.

EXAMPLE 5

N-methyl-4,4'-bipyridinium iodide (monoquat) was synthesized by a standard technique. 1.13 gram monoquat was dissolved in 70 mls. acetonitrile and 25 mls. DMF. 9.0 mls. 1,4-dibromobutane was added and the solution was refluxed overnight. It was then cooled, the precipitate was filtered, washed with acetone and dried. The mixed bromo,iodo salt of the resulting viologen was dissolved in water, filtered and precipitated as the hexafluorophosphate (PF_6) salt through addition of

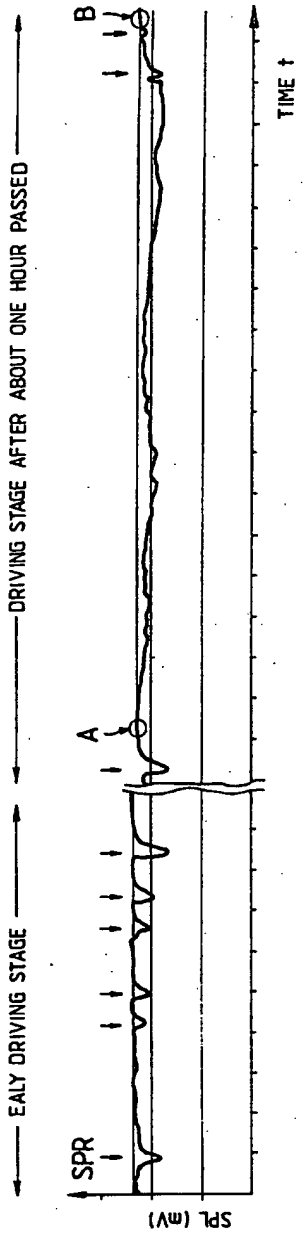

EALY DRIVING STAGE
DRIVING STAGE AFTER ABOUT ONE HOUR PASSED
SPR
A
B
TIME t
SPL (mV)

FIG. 3

ammonium hexafluorophosphate. This was filtered and dried in vacuum.

0.50 gram PVP and 1.50 gram viologen were dissolved in 60 mls. of DMF and heated to 68° C. overnight. Then about 2grams of 2-bromoethylamine hydrobromide was added to the war solution. After about 90 minutes, the DMF was decanted from the precipitated polymer, and the polymer was dissolved in water, filtered and precipitated as the PF_6 salt. This was dried, then redissolved in DMF containing 2-bromoethylamine hydrobromide. After further warming at 68° C. overnight, much of the polymer had precipitated. Tetrabutyl ammonium bromide was added to precipitate the rest which was filtered and washed with methylene chloride. The very hygroscopic polymer was stored in a vacuum dessicator. The approximate structure of this polymer (Polymer D) is shown in FIG. 2.

Three solutions were made up in 10 mM HEPES buffer at pH 8:1:

Solution 1 was 5 mg/ml Polymer D

Solution 2 was about 5 mg/ml nitrate reduces

Solution 3 was 2.7 mg/ml PEG-DGE

25 microliters of solutions 1 and 2 were thoroughly mixed with 10 microliters of solution 3. 4 microliters of this mixture was applied to the surface of a 3 mm diameter glassy carbon disk electrode and cured overnight in a vacuum at room temperature. Upon exposure of this electrode to a deaerated solution containing 25 mM nitrate, a reduction current density of 22.6 microA/cm^2 was recorded at a potential of −0.8 volts relative to the SCE reference. Under the same conditions in the absence of nitrate ion the background current density was 7.0 microA/cm^2.

EXAMPLE 6

4'-Methyl,4'-(4-bromobutyl) bipyridine, made from the monolithium salt of dimethylbipyridine and 1,4-dibromobutane, was used as a starting material. 1.11 gram of this was dissolved in 50 mls. of ethylene diamine and warmed to about 80° C. for 2.5 hours. The solvent was then removed under vacuum, the residue was dissolved in ethyl acetate and the product was extracted into aqueous solution at pH 5.1. The aqueous solution was washed with methylene chloride. It was then made basic and the product was extracted into methylene chloride, washed with water, dried and evaporated.

190 mgs of the resulting 4-methy1,4'-(butylaminoethylamine) bipyridine was dissolved in 4 mls. DMF and 144 mgs of K_2OsCl_6 was added and refluxed for 1 hour. Water and dilute HCl were added to the DMF solution, it was filtered and the product was precipitated by the addition of ammonium hexafluorophosphate. The product was dried under vacuum. The structure of this compound is shown in FIG. 2 (Polymer G).

A 3mm glassy carbon disk electrode was made by applying 3 microliters of 5 mg/ml glucose oxidase in 10 mM HEPES buffer pH 8.1, 1 microliter of 2.7 mg/ml PEG-DGE in the same buffer and 3 microliters of 10 mg/ml Polymer G in acetonitrile. The electrode was cured overnight in vacuum. Upon exposure to a solution containing a high concentration of glucose (≧60 mM), this electrode exhibited a current density of 2.1 microA/cm^2 when held at a potential of 0.15 V relative to the SCE reference. The background current density in the absence of glucose was 0.84 microA/cm^2 at the same potential.

This invention has been disclosed in connection with specific embodiments. However, it will be apparent to those skilled in the art that variations may be undertaken without departing the spirit and scope of the invention.

What is claimed is:

1. An electrode having a surface coated with a film, the film comprising:

a crosslinked polymer having multiple redox centers; and

a redox enzyme bound within the crosslinked polymer,

wherein the crosslinked polymer provides electrical contact between the electrode and the enzyme.

2. The electrode of claim **1**, wherein the crosslinked polymer includes a plurality of transition metal complexes, each complex having a plurality of organic ligands.

3. The electrode of claim **2**, wherein the transition metal comprises osmium.

4. The electrode of claim **1**, wherein the crosslinked polymer includes a plurality of organic redox centers.

5. An amperometric biosensor having an electrode as recited in claim **1**.

6. The amperometric biosensor of claim **5**, wherein the electrode is capable of selectively sensing one of the following biochemicals: glucose, lactate, glycerol-3-phosphate, L-amino acids, or D-amino acids.

7. The amperometric biosensor of claim **5**, wherein the electrode is capable of selectively sensing nitrate.

8. A bioelectrosynthesizer having an electrode as recited in claim **1**.

9. The electrode of claim **1**, wherein the redox enzyme is covalently bonded to the crosslinked polymer.

10. An electrode having a surface coated with a film, the film comprising:

a hydrophilic cross-linked polymer having multiple redox centers; and

a redox enzyme bound within the cross-linked polymer, wherein the cross-linked polymer provides electrical contact between the electrode and the enzyme.

11. An electrode made of a material selected from the group consisting of gold, platinum, glassy carbon and graphite, said electrode having a surface coated with a film, the film comprising:

a cross-linked polymer having multiple redox centers; and

a redox enzyme bound within the cross-linked polymer, wherein the cross-linked polymer provides electrical contact between the electrode and the enzyme.

12. The electrode of claim **11**, wherein the resulting electrode exhibits current densities in excess of 10 micro Amps/cm^2 in the presence of substrate.

13. The electrode of claim **12**, wherein the resulting electrode exhibits current densities in excess of 100 micro Amps/cm^2 in the presence of substrate.

14. The electrode of claim **13**, wherein the resulting electrode exhibits current densities in excess of 1000 micro Amps/cm^2 in the presence of substrate.

15. An electrode having a surface coated with a film, the film comprising:

a cross-linked polymer having multiple redox centers; and

a redox enzyme bound within the cross-linked polymer, wherein the cross-linked polymer provides electrical contact between the electrode and the

FIG. 1

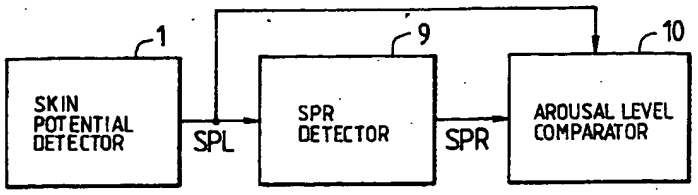
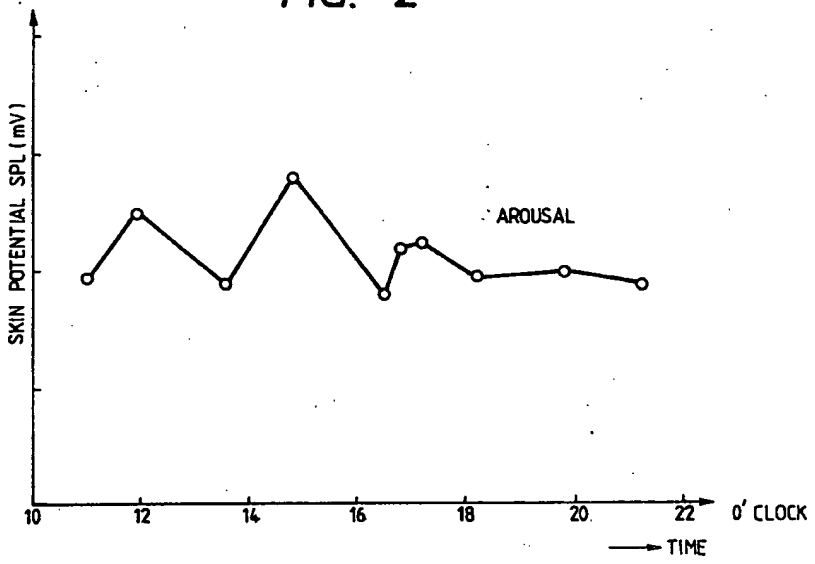

1
9
10
SKIN POTENTIAL DETECTOR
SPL
SPR DETECTOR
SPR
AROUSAL LEVEL COMPARATOR

FIG. 2


SKIN POTENTIAL SPL (mV)
AROUSAL
10
12
14
16
18
20
22
0' CLOCK
TIME

enzyme, and wherein the resulting electrode has an operating potential in the range of 0.1V to 0.5V versus the Standard Calomel Electrode.

16. An electrode having a surface coated with a film, the film comprising:

a cross-linked polymer having multiple redox centers; and

a redox enzyme bound within the cross-linked polymer, wherein the cross-linked polymer provides electrical contact between the electrode and the enzyme, and

wherein the resulting electrode exhibits current densities in excess of 10 micro Amps/cm^2 in the presence of substrate.

17. The electrode of claim 16 wherein the resulting electrode exhibits current densities in excess of 100 micro Amps/cm^2.

18. The electrode of claim 17 wherein the resulting electrode exhibits current densities in excess of 1000 micro Amps/cm^2.

* * * * *

United States Patent [19]

Yoshimi et al.

[11] **Patent Number: 4,928,090**

[45] **Date of Patent: May 22, 1990**

[54] **AROUSAL LEVEL JUDGING APPARATUS AND METHOD**

[75] Inventors: **Tomohisa Yoshimi**, Gamagori; **Satoru Kodama**, Oobu; **Takeshi Yoshinori**, Chiryu; **Masahiko Ito**, Nagoya, all of Japan

[73] Assignee: **Nippondenso Co., Ltd.**, Kariya, Japan

[21] Appl. No.: **281,074**

[22] Filed: **Dec. 8, 1988**

[30] **Foreign Application Priority Data**

Dec. 9, 1987 [JP] Japan 62-312972
Jul. 18, 1988 [JP] Japan 63-178793

[51] **Int. Cl.**[5] **G08B 21/00**

[52] **U.S. Cl.** **340/575**; 128/734; 340/576

[58] **Field of Search** 340/576, 575; 128/734

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,088,125 5/1978 Forgione et al. 128/734
4,725,824 2/1988 Yoshioka 340/576

OTHER PUBLICATIONS

"Feedback Control of the Level of Arousal Using Skin Potential Level as an Index", by Chiaki Nishimura et al.; Ergonomics 1985, vol. 28, No. 6, pp. 905–913.

"Estimation of the Level of Arousal by Skin Potential and its Application to Sleep Control", by Chiaki Nishimura et al.; MBE 83–27; pp. 53–60.

"Evaulation of the Arousal Level in Motorcar Driving Using Skin Potential Level I: Changes in Skin Potential Level Under Various Driving Conditions", by Chiaki Nishimura et al.; from Ningen Kogaku, vol. 23, No. 2('87), pp. 103–110.

"Evaluation of the Arousal Level in Motor Driving Using Skin Potential Level II: Its Effectiveness Compared with Other Electrophysiological Quantities", by Chiaki Nishimura et al.; from Ningen Kogaku, vol. 23, No. 2('87); pp. 111–118.

"Biofeedback of Skin Potential Level for Sleep Control", by Chiaki Nishimura et al.; from Kenkyu, vol. 18, No. 5 (Sep. 1980); pp. 327–333.

"Feedback Control of the Level of Arousal in Motor Driving", by Chiaki Nishimura et al.; from Biofeedback Kenkyu (1984).

Primary Examiner—Glen R. Swann, III

Attorney, Agent, or Firm—Cushman, Darby & Cushman

[57] **ABSTRACT**

An arousal level judging apparatus has a skin potential level (SPL) detection device, a potential change detection device for detecting occurrance and convergence of a pulse of the signal from the SPL detection device, the pulse being caused by skin potential response (SPR), a storing device for storing the SPL value as a reference value, and a comparator for comparing SPL with the reference value to detect a SPL decrease to detect an arousal level decrease of a driver, etc. The reference value is renewed at every occurrance of SPR. When the SPL value indicates that the arousal level of the driver has been decreased, an alarm is caused to wake the driver up.

12 Claims, 11 Drawing Sheets